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Intestinal Adaptation to Repeated Exposure of Flavonoid-rich Foods: In Vitro and Clinical Data

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INTESTINAL ADAPTATION TO REPEATED EXPOSURE OF FLAVONOID-RICH FOODS: IN VITRO AND CLINCAL DATA

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To my Grandpa, James (Jim) P. Van Hook, Ph.D., who would have loved to read this dissertation. His many discussions about science and his continuous support inspired my drive to pursue research.

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LIST OF ABBREVIATIONS

AUC	Area under the curve
BMI	Body mass index
C	Catechin
C_{max}	Maximum plasma concentration
CNTL	Control
COMT	Catechol-O-methyltransferase
Cy	Cyanidin
EC	(-)-Epicatechin
EGCG	(-)-Epigallocatechin-3-gallate
Glucur	Mlucuronide
MCT	Monocarboxylate transporter
MRP	Multidrug-resistance protein
PK	Pharmacokinetic
P-gp	Permeability-glycoprotein
SGLT1	Sodium dependent glucose transporter 1
SULT	Sulfotransferase
UGT	UDP-glucuronyltransferase

ABSTRACT

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Interest in application of flavonoids for chronic disease prevention has grown significantly, but the low oral bioavailability of these compounds from acute doses is commonly highlighted as a limitation when considering their biological significance. Still, the impact of broad dietary patterns such as repeated exposure on flavonoid's absorption, metabolism, and eventual efficacy is critical to consider since evidence suggests that their bioavailability may be enhanced with repeated exposure. To fill this gap in knowledge, this dissertation will focus on three major areas including characterization of flavonoid metabolites, in addition to use of *in vitro* models and clinical work to test the effect of repeated exposure on flavonoid bioavailability.

Though flavan-3-ols undergo Phase II metabolism in humans and rodents, researchers have generally not been able to utilize fully characterized standards for these metabolites. Thus, collaborators synthesized flavan-3-ols metabolites after which liquid chromatography/time-of-flight mass spectrometry (LC-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy were used to characterize their structure and match the synthesized metabolites to those found in rodent plasma. To explore changes

occurring in the upper small intestine from flavonoid repeated exposure, Caco-2 cells were differentiated in the presence of isolated flavan-3-ols, green tea, grape seed, or blackberry extracts. EGCG and EC pretreatment altered formation rate of Phase II metabolites, in addition, green tea and grape seed extract pretreatment both resulted in increased flavan-3-ol transport. In contrast, blackberry extract pretreated monolayers displayed decreased transport of phenolic compounds. Finally, alterations in mRNA expression of select transport and metabolizing genes were observed in cells pretreated with blackberry extract. To determine if flavonoid absorption changes with repeated exposure to blackberry in humans, a controlled feeding study was performed to assess the effect of three-week daily blackberry exposure on flavonoid pharmacokinetics. The results showed increased plasma AUC of peonidin glucoside after blackberry treatment in lean volunteers. Accumulation of total anthocyanins in urine was greater in the lean group after blackberry exposure. This difference may be driven by increased Phase II anthocyanin metabolites since there was greater accumulation of anthocyanin metabolites in urine in the lean group after blackberry exposure.

Taken together, these data suggest that the small intestine may be a key regulator of the observed adaptive phenomena occurring in *vivo*. These results demonstrate that there is both differential transport, absorption, and metabolism of flavonoids, including select flavonoids and phenolic acids, after repeated exposure to flavonoid-rich blackberry and that this response appears to differ with BMI. These studies provide a basis for future work on the effect of chronic flavonoid exposure on their bioavailability and metabolism in a range of interventions.

CHAPTER 1. REVIEW OF THE LITERATURE

As part of the manuscript "Altered Transport and Metabolism of Phenolic Compounds in Obesity and Diabetes: Implications for Functional Food Development and Assessment,"

BW Redan[1], JA Novotny[2], and MG Ferruzzi[1,3], in review, Advances in Nutrition.

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1.1 Introduction

Plant-derived phenolic compounds are a subclass of phytochemicals characterized by the presence of one or more phenol moieties, and their consumption is associated with a reduced risk of chronic disease. While evidence has primarily been drawn from the epidemiological associations between foods rich in phenolics and disease-risk outcomes (Zamora-Ros et al., 2013a), data from in vitro studies (Tenore et al., 2013), animal models (Tian et al., 2013), and more crucially clinical trials have begun to substantiate these epidemiological associations (Hooper et al., 2012). While results are not fully conclusive (Jia et al., 2010), these data generally support the notion that plant-

derived phenolic compounds likely impart benefits to humans. As a result, desire to expand application of phenolic compounds as a disease-preventative agent is gaining momentum, in part, due to the cost-effective nature of dietary prevention strategies relative to therapeutic approaches. This concept has been translated into formulation of phenolic compounds in functional foods and dietary supplements for the broader public.

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Phenolic compounds can be broadly divided into flavonoid and non-flavonoid derivatives (Del Rio et al., 2013) (see **Figure 1**). The flavonoid class encompasses six major subclasses, each consisting of a three ring structure designated as A, B, and C. The main subclasses are flavonols, flavones, isoflavones, flavanones, anthocyanins, and

flavan-3-ols, which are all considered polyphenols because of their multiple phenol moieties. The flavan-3-ol (-)-epicatechin, which is found in high amounts in cocoa, grape seed, and to a lesser extent in apples and tea, has received particular attention due to is presence in high quantities in certain chocolates combined with chocolate's general popularity. Non-flavonoid phenolics include phenolic acids, stilbenoids, and lignins. One of the most common dietary non-flavonoids is the phenolic acid gallic acid, present in grapes and other fruits. Phenolic compounds naturally occur in either free or glycosylated forms (Del Rio et al., 2013), which is significant since the glycoside addition can modulate the solubility, chemistry, and bioavailability of individual phenolic compounds (Murota et al., 2010; Okabe et al., 2011).

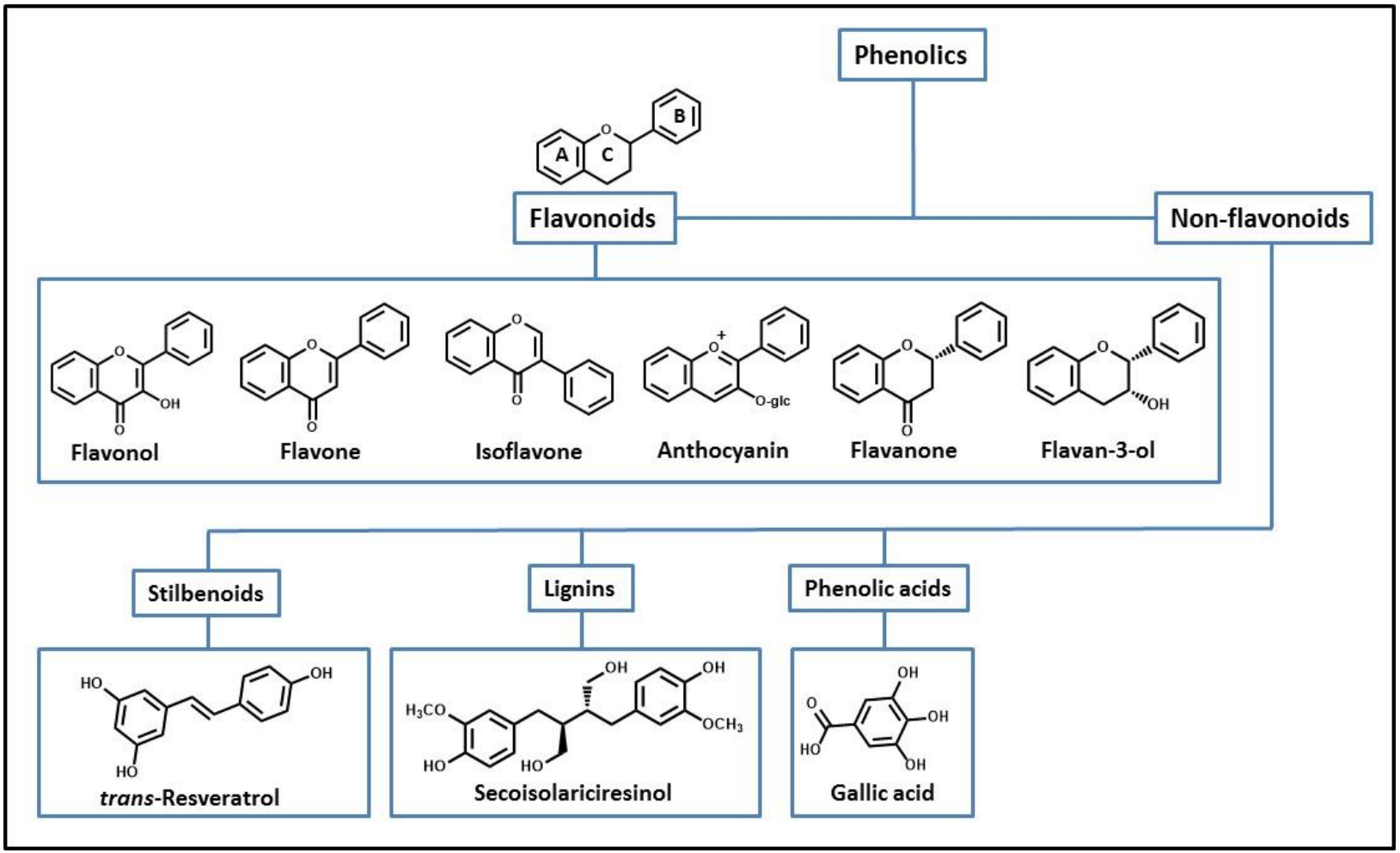

Figure 1. Flowchart of the main classes of phenolics with key examples of their chemical structure.

The phenolic contents of common foods and beverages have been systematically reviewed and documented in several publically available databases, including USDA's Nutrient Data Laboratory Flavonoid Database and INRA's Phenol-Explorer (Bhagwat et al.; Gu et al., 2004; Neveu et al., 2010; Pérez-Jiménez et al., 2010a, 2010b). The predominate dietary sources of phenolics in the Western diet are generally accepted to be fruits, vegetables, tea, wine, and cocoa products, with mean dietary intakes for flavonoids ranging from a low of approximately 250 mg/day for U.S. adults (Sebastian et al., 2015), 250-300 mg/day for Greek men and women (Zamora-Ros et al., 2015), 320 mg/day for Korean adults (Jun et al., 2016), to a high of 1,000 mg/day for British adult populations (Zamora-Ros et al., 2015).

The diverse mechanisms proposed for the biological activities of phenolic compounds are dependent on their structure and cellular target. Regardless of the structure, dietary source of the phenolic compound, or specific mechanism by which it may impart its biological activity, the ultimate efficacy of dietary phenolics is dependent on the ability of the compounds to be absorbed intact or as a biologically relevant metabolite, and then transported to target tissues where they can exert their biological activity (Ferruzzi, 2010). The bioavailability of phenolics is therefore a critical factor to consider when evaluating the efficacy of a food/supplement or designing products for at-risk populations. In addition to these factors, disease states such as obesity or diabetes that are common in industrialized countries (Malik et al., 2013) can impact the bioavailability of nutrients and potentially phytochemicals (Xiao and Högger, 2014), which may be related to the ability of these diseases to modulate gut function (Horowitz

et al., 2002; Meyer-Gerspach et al., 2014), alter regulation of xenobiotic metabolizing enzymes (Xiao and Högger, 2014), or perturb the gut microbial community critical to the metabolism of phenolics (Hartstra et al., 2015).

This review will specifically focus on the effect of obesity and diabetes on the bioavailability and bioactivity of dietary phenolics. Some outstanding questions on absorption and metabolism of phenolic compounds include: *(1) Why are phenolic compounds potentially more effective in improving physiological conditions in individuals who are lean or non-diabetic? (2) Are the absorption, metabolism, and transport of phenolic compounds altered in individuals with obesity or diabetes? (3) If so, what mechanism may be effective to target in order to increase biological activity of phenolic compounds in obese or diabetic individuals?*

1.2 Overview of the Absorption, Metabolism, and Transport of Phenolic Compounds

Preventative or therapeutic potential of phenolic compounds is highly dependent on their ability to reach target tissues in a form that can exert biological function. Multiple steps in this process include absorption, metabolism, and transport, in both the upper and lower gastrointestinal tract (Neilson and Ferruzzi, 2011) (Summarized in **Figure 2**). The first key step in the absorptive process is the release of phenolics from the plant/food matrix into the soluble and bioaccessible fraction in the gut lumen. Soluble phenolic forms are then actively and passively transported from the lumen into the enterocyte, but then can be effluxed back out into the lumen by the action of ATP-binding cassette (ABC) transporters. Within the enterocyte, phenolics may also be

metabolized prior to transport across the serosal side and into the portal vein for circulation to the liver, where further metabolism may occur prior to systemic circulation, excretion into bile/urine, or distribution to other tissues. Unabsorbed phenolics or those secreted back into the gut lumen are carried to the lower gut and made available to microbiota for catabolism into low molecular weight compounds, including several previously characterized phenolic metabolites (Calani et al., 2012). Since the broader aspects of absorption, metabolism and target tissue profiles have been the subject of several reviews (Clifford et al., 2013; Del Rio et al., 2010, 2013; Manach et al., 2005; Williamson and Manach, 2005), below are only key aspects of this process, focusing on upper intestinal absorption and metabolism as they rely on both inducible and non-inducible systems (Croom, 2012) that may be impacted by both health status and previous exposure to phenolics. Factors in addition to the metabolizing systems that are potentially affected by disease state, such as gut microbiota, will not be covered extensively but will be discussed in a later section.

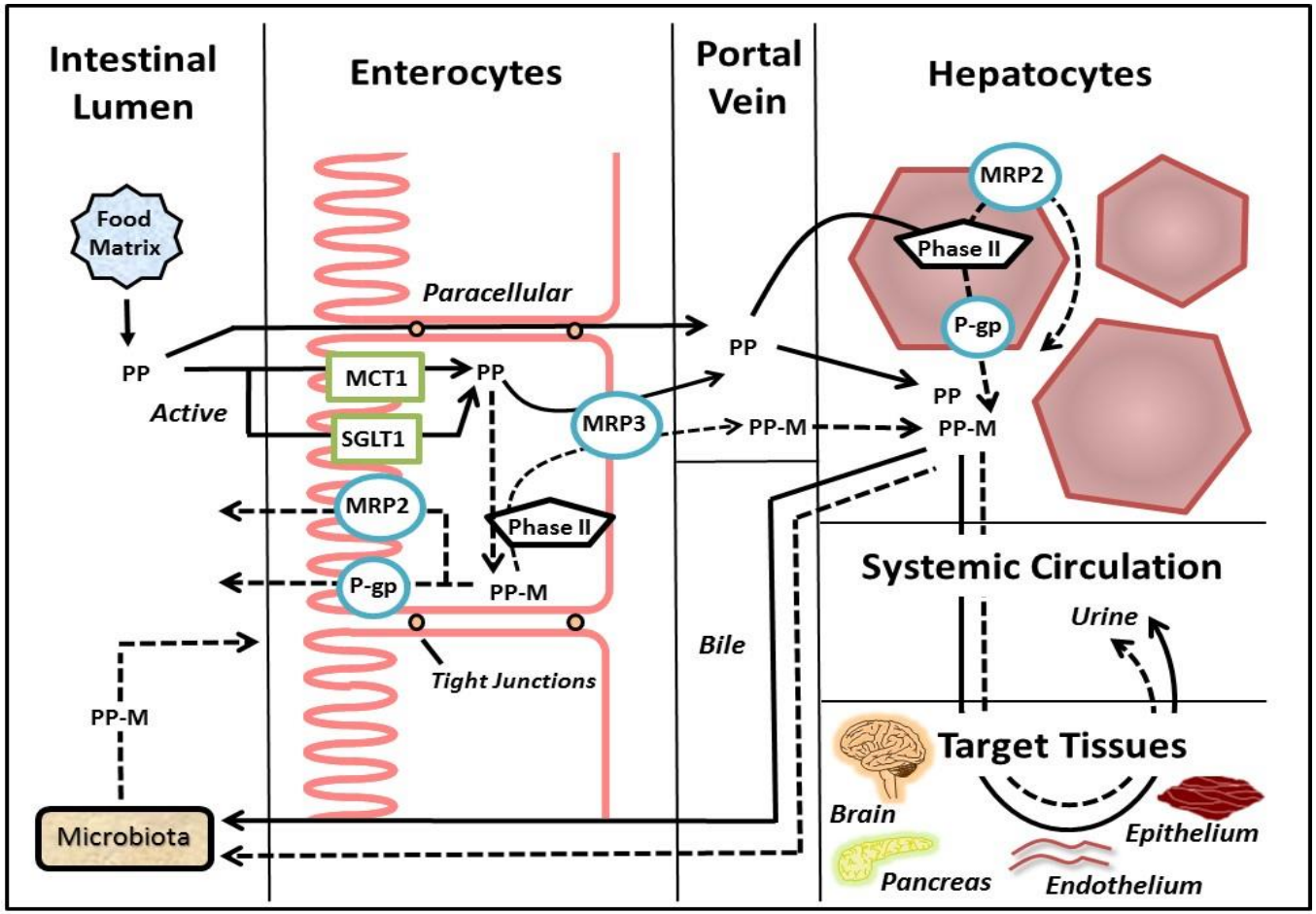

Figure 2. Schematic of the key molecular processes involved in the transport and metabolism of (poly)phenolic compounds (PP). PP-M, (poly)phenolic metabolites; MCT1, monocarboxylate transporter1; SGLT1, sodium dependent glucose transporter 1; P-gp, P-glycoprotein; Phase II, Phase II metabolizing enzymes; MRP3, multidrug-resistance protein 3; solid arrow, pathway of native phenolic compounds; dashed arrow, pathway of phenolic metabolites.

1.3 Absorption of Phenolic Compounds

Absorption of phenolics in their dietary forms generally takes place in the small intestine, although select phenolics may be absorbed in the stomach (anthocyanins) and extensively in the lower intestine/colon (products of microbial catabolism). Uptake from the intestinal lumen by enterocytes can occur for either the glycoside or aglycone forms of phenolics (Del Rio et al., 2013). Glycosides of many phenolic compounds are thought to be translocated from the gut lumen to the enterocyte by transporters, including sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2). Since glycosidic forms are effluxed back into the intestinal lumen after uptake by the enterocyte (Del Rio et al., 2013), absorption efficiency is believed to be increased by first hydrolyzing the carbohydrate moiety attached to *O*-linked compounds through the action of enzymes produced the intestinal brush border or those synthesized by gut microbiota. The aglycone form of phenolics can then enter the enterocyte through active uptake by transporters such as monocarboxylate transporter 1 (MCT1) or to a certain extent by passive diffusion (Vaidyanathan and Walle, 2003). Once within the enterocyte, phenolics can then be metabolized (described below) or effluxed back into the intestinal lumen by transporters such as multidrug resistance-associated protein 1 or 2 (MRP1/2).

1.4 Metabolism of Phenolic Compounds

Phenolics are largely transformed and transported via xenobiotic metabolizing systems expressed extensively in intestinal and hepatic tissues (Lampe and Chang, 2007),

but also in other tissues and compartments including the blood-brain barrier (Campos-Bedolla et al., 2014; Ouzzine et al., 2014). Absorbed phenolic compounds undergo Phase II conjugation to produce metabolites that are less chemically reactive and either more hydrophilic or hydrophobic compared to the parent compound, thereby facilitating transport processes leading to excretion by the Phase III system through bile/feces or urine (see **Figure 3**). Since the extent to which individual phenolics are biotransformed may result in alteration of specific biological activity (Lambert et al., 2007; Monagas et al., 2010; Spencer et al., 2004), factors that ultimately affect metabolism of phenolics are believed to have significant impact on their potential to exert protective therapeutic effects at target tissues.

The methylation of phenolics driven by catechol-*O*-methyltransferase (COMT) results in a more hydrophobic and less chemically reactive species (Croom, 2012). This increased hydrophobicity may then increase the association of phenolics with key transporters on the cell membrane, altering their transport across the enterocyte or other cell types (Walle, 2007), leading to the potential for accumulation of methylated derivatives in certain tissues such as brain (Chen et al., 2015; Ferruzzi et al., 2009; Wang et al., 2012). However, methylation generally decreases phenolic antioxidant capacity *in vitro* (Dueñas et al., 2010; Ishimoto et al., 2012) and thus may also affect bioactivity. For example, while methylation of the tea flavan-3-ol epigallocatechin-3-gallate (EGCG) impairs its ability to decrease tumor proliferation (Lambert et al., 2007), the flavone chrysin has greater anti-inflammatory ability in the human intestinal cell line Caco-2 when methylated (During and Larondelle, 2013). In contrast, methylated epicatechin

glucuronide derivatives were observed to have greater ability to enhance long-term potentiation in hippocampal slices relative to simple glucuronides (Wang et al., 2012). The effect of the methylation transformation on bioactivity is a subject of significant debate and appears to be specific to the class of phenolic being metabolized.

Sulfotransferases (SULT) conjugate anionic sulfate groups to phenolic moieties to produce a more hydrophilic species (Peters et al., 1990), resulting in either a more or less bioactive species depending on the cellular target (Zhang et al., 2004). For example, sulfate metabolites of the grape phenolic resveratrol, in contrast to the resveratrol aglycone and its glucuronide, are not effective in decreasing lipid content of differentiated adipocytes, while sulfate metabolites have been reported to be effective in decreasing lipid content of maturing adipocytes (Lasa et al., 2012).

Glucuronidation conjugates glucuronic acid to phenolic compounds through the action of UDP-glucuronyl transferase (UGT) to yield a more hydrophilic species that can be efficiently eliminated (Sinnott, 1990). Glucuronidation may affect binding of phenolics to specific cellular targets, as in the case of resveratrol, but overall this biotransformation does not seem to affect resveratrol's *in vitro* antioxidant potential (Lu et al., 2013). Additionally*, in vitro* models show that glucuronidation does not affect quercetin's estrogenic activity (Ruotolo et al., 2014). Though this metabolite in certain cases does not seem to have diminished bioactivity compared to the parent compound, the increase in its water solubility increases its potential for excretion and thus may impact bioactivity by limiting the half-life and concentrations in target tissues.

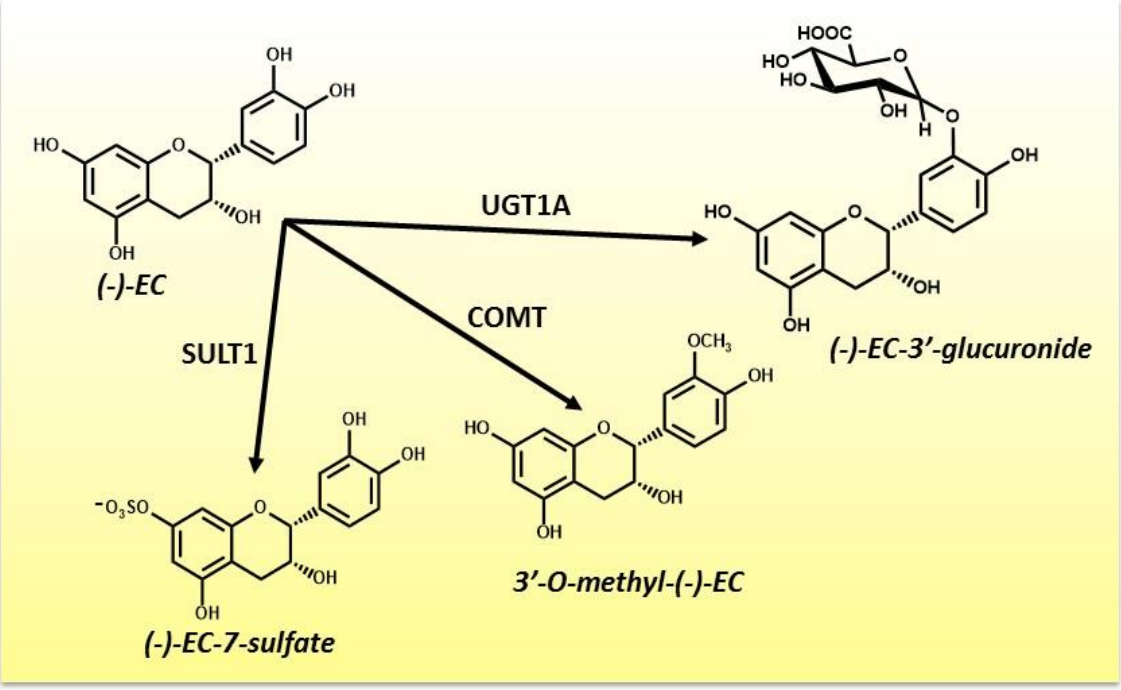

Figure 3. Summary of Phase II metabolism. Phase II metabolism of the flavan-3-ol (-)-epicatechin (EC) can produce metabolites that differ in bioactivity due to a change in chemical reactivity and hydrophobicity compared to the parent compound. Conjugation can occur at other and additional R-OH positions than those as displayed in the figure. Abbreviations: UGT: UDP-glucuronyltransferase; COMT: catechol-O-methyltransferase; SULT: sulfotransferase.

1.5 Transport of Phenolic Compounds

After phenolics are conjugated by the Phase II metabolizing system, they can be transported to excretory pathways or systemic circulation leading to exposure to target tissues. Efflux can occur at various sites along the gut, liver, and kidney by transporters such as permeability glycoprotein (P-gp), also called multidrug resistant protein 1 (MDR1) (Walle, 2004). P-gp has a net effect of lowering apparent oral bioavailability of flavonoids, such as epicatechin-3-gallate (ECG), by promoting efflux from the enterocyte to the intestinal lumen (Vaidyanathan and Walle, 2003). However, phenolic interactions

with transporters may be competitive, and certain phenolic compounds that have a higher affinity for these MDR transporters may serve to enhance absorption of others. For example, gallated catechins have been shown to enhance bioavailability of other phenolic compounds in tea (Tagashira et al., 2012), which may be related to their ability to inhibit P-gp by binding to its active site (Qian et al., 2005). It is therefore important to consider phenolics in the context of a whole-food system containing various types of phenolics and other compounds, such as tea, which may lead to an overall increased absorption of targeted phenolics in the same system compared to isolated compounds.

There is an important distinction to make in regard to efflux transporters, as they can either increase or impair delivery of phenolics to target tissues. In enterocytes, MRP2 localizes to the luminal side (Leslie et al., 2001), MRP3 to the serosal position, while MRP1 can be located on both sides (Del Rio et al., 2013; Estudante et al., 2013). Due to this polarization, MRP 1 or 2 can both facilitate efflux of compounds into excretory pathways in contrast to MRP3, which can act to enhance transport of these compounds to target tissues by increasing flux into the portal vein. Since the Phase II and efflux systems may reduce the activity of phenolic compounds, they may be a potential target to assess if affected in diseased conditions.

1.6 Metabolic Regulation of Xenobiotic Transport and Metabolism: Potential Implications for Phenolics

Since xenobiotic transport and metabolism are dependent on normal metabolic regulation, it can be expected that diseases such as obesity or diabetes may in fact alter

these systems and by extension impact bioavailability and effectiveness of phenolics. Several clinical trials that will be discussed have reported minimal to no improvements in physiological markers among obese or diabetic subjects given phenolic-rich foods, while there are improvements in groups without these conditions. It is therefore logical to assume that phenolics may not be as effective in improving health outcomes if a disease state such as obesity or diabetes is present. We will further review studies demonstrating that high therapeutic doses of phenolics are often needed to elicit changes in the health status of obese or diabetic individuals, further suggesting alterations in the absorption and metabolism of these compounds that results in their lowered effectiveness.

1.7 Clinical Trials Comparing Efficacy of Phenolic Compounds in Healthy vs. Obese or Diabetic Populations

Several studies suggest differential effects of phenolic compounds in lean versus obese or diabetic populations in regards to cardiovascular function. Banini *et al.* (Banini et al., 2006) found that consumption of dietary grape phenolics produced a differential response between diabetic and non-diabetic individuals on markers of cardiovascular health and glucose control. To assess this difference, diabetic and healthy individuals were given either 150 mL of juice, wine, or a dealcoholized wine beverages made from Muscadine grapes for 28 days. Non-diabetic controls in this study had a BMI range from about 27-30 kg/m^2 (overweight), and the diabetic BMI range was from about 35-40 kg/m^2 (obese). Comparing changes in lipid profiles, grape juice consumption significantly

lowered triglyceride levels in only the non-diabetic participants. Diabetics consuming the dealcoholized wine had decreased insulin levels at the end of the study compared to baseline, however this reduction may simply have been from consuming less carbohydrate and not necessarily due to phenolic treatment. One strength of this study is that it tested standard wine along with dealcoholized wine, which is significant since alcohol can be a potential confounding factor by effecting health outcomes and/or bioavailability (Brien et al., 2011; Dragoni et al., 2006). While the authors did not report a specific phenolic composition of the juice, an analysis elsewhere suggest that 150 mL of the wine could contain approximately 443 mg total phenolics, including 6 mg of gallic acid (Otaolaurruchi et al., 2007). While this was a lower dose compared to other studies reviewed here, lipid profile still improved in the overweight, non-diabetic subjects, but the diabetic group did not show these improvements.

Similarly, Davison *et al.* (Davison et al., 2008) found that higher doses of cocoa phenolics are needed to produce a response in cardiovascular markers and glucose control in overweight and obese individuals. Participants in this study had an average baseline BMI of 33.5 kg/m^2 and were given a cocoa beverage with either low or high levels of flavan-3-ols (36 versus 902 mg per day). This treatment occurred over a period of 12 weeks, with measurement tests conducted at baseline, 6, and 12 weeks. Measurements at 12 weeks showed that only the high-dose group had an increase in flow-mediated dilatation, a marker of cardiovascular function, along with improved markers of insulin resistance. A strength of this study is the relatively prolonged treatment time period, in conjunction with the low and high doses to demonstrate the

point at which a physiological response takes effect. In this study, there was not a normal weight group to directly compare the results and the relatively high dose of phenolics (902 mg of flavan-3-ols) with more relevance to supplemental treatments rather than dietary exposure. Though this study does not point specifically to an underlying mechanism explaining why a much higher dose was required to elicit a response compared to other studies, these data suggest that a higher dose of flavan-3-ols is needed to be effective in promoting cardiovascular health and normal insulin markers in overweight and obese individuals.

Almoosawi *et al.* (Almoosawi et al., 2012) reported that cocoa phenolics elicit a differential response in cardiovascular and glucose markers in overweight and obese individuals versus normal weight participants. In this study, normal weight (BMI 18.5-24.9 kg/m^2) and overweight plus obese (BMI ≥ 25 kg/m^2) participants were given 20 g of a cocoa confection containing 500 mg total phenolics (19 mg from flavan-3-ols epicatechin and catechin) daily for 4 weeks. The study used a crossover design, and the control product consisted of participants receiving a sham cocoa confection with its phenolics removed. The overweight/obese group had reductions in both systolic and diastolic blood pressure in addition to blood glucose levels at the conclusion of the study. The normal weight group only had a reduction in systolic blood pressure, but this was likely due to the normal blood pressure and glucose levels among individuals in the lean group. This study used a well-designed control confection that matched lipid profiles while excluding phenolics. As cocoa butter fatty acids alone have been reported to improve cardiovascular markers (Mursu et al., 2004), this suggests the direct

difference in phenolic impacts. One reason for observing the positive results in both the overweight and obese group is their categorization in the study. If the two groups were separated in the study, statistical analysis may have shown overweight participants responding to the treatment but not the obese. Though some research has not been able to confirm a significant relationship between BMI and effective dose of polyphenols (Almoosawi et al., 2010), the authors still found the results unexpected since they conflict with some previous research suggesting lowered efficacy of polyphenol treatment in obese populations. Although an effect was observed in this study on markers of glucose control and cardiovascular health in the overweight and obese population, it still leaves questions, including why studies show responders and non-responders to phenolic treatments. This question may have been better answered if the overweight and obese individuals were separated into their own groups, as only those with BMI levels in the obese range may be negatively affected.

Combined, these studies suggest that phenolics may have differential effects in lean versus obese or diabetic populations in regards to cardiovascular function. However, it is not clear as to the underlying reasons for this difference. It is logical that differences in absorption and metabolism of phenolics between healthy and obese or diabetic individuals would be responsible in part for these observations. With lower absorption, higher doses may be required to achieve appropriate circulating and tissue concentrations to elicit a physiological response. While the studies described did not directly measure absorption of phenolics, evidence from *in vitro*, animal, and other clinical studies provide more insight on this potential effect.

1.8 Pharmacokinetic Analyses in Animals Models Suggest Obesity and Diabetes Affect Xenobiotic Transport and Metabolism

Several studies have suggested that obese or diabetic conditions may lead to a fundamental alteration in phenolic xenobiotic metabolism and transport systems critical for delivering the effect of this bioactive. **Table 1** summarizes the pre-clinical animal models showing that both obesity and diabetes affect the xenobiotic metabolizing and transporting systems critical for delivery of phenolics (Lee et al., 2010). Many of these studies sought to determine differences in individual pharmacokinetic parameters of phenolics and other compounds in these diseased conditions relative to healthy models to help elucidate underlying mechanisms for the varying physiological responses to these compounds.

Table 1. Summary of pre-clinical pharmacokinetic studies.

Study and Reference	Model and Health Status	Source	Time (hr)	Compound	Dose ($mg \cdot kg^{-1}$)	AUC ($ng \cdot hr \cdot mL^{-1}$)	C_{MAX} ($ng \cdot mL^{-1}$)	T_{MAX} (hr)	AUC/dose ($ng \cdot hr \cdot mL^{-1} \cdot mg^{-1}$)	C_{MAX}/dose ($ng \cdot mL^{-1} \cdot mg^{-1}$)
Liu, 2010 (64)	Male Sprague-Dawley rats	Baicalin, i.v.	10	Baicalin	12	18,000	–	–	1,500	–
	STZ-induced diabetic rats					11,200	–	–	930	–
	Male Sprague-Dawley rats	Baicalin, oral	24		200	48,500	3,390; 3,860	0.2; 8.4	240	17; 19
	STZ-induced diabetic rats					100,800	5,740; 8,630	0.2; 8.8	500	29; 43
Yu, 2010 (66)	Male Sprague-Dawley rats	*Coptidis Rhizomea* extract	8	Jatrorrhizine	34	290	150	2.88	8.5	4.4
	STZ-induced diabetic rats					2,150	1,050	1.88	63.2	30.9
Liu, 2012 (75)	Male Wistar rats	Mangiferin	∞	Mangiferin	400	3,820	720	0.73	9.6	1.8
	STZ-induced diabetic rats					8,950	2,000	2.25	22.4	5.0
Kang, 2010 (86)	Male Sprague-Dawley rats	Liquiritigenin, i.v.	12	Liquiritigenin	20	6,400	–	–	320	–
				Metabolite 1		13,100	223	3 min	655	11.2
				Metabolite 2		12,300	410	3 min	615	20.5
	STZ-induced diabetic rats			Liquiritigenin		5,910	–	–	296	–

Table 1, con'd. Summary of pre-clinical pharmacokinetic studies.

Study and Reference	Model and Health Status	Source	Time (hr)	Compound	Dose ($mg \cdot kg^{-1}$)	AUC ($ng \cdot hr \cdot mL^{-1}$)	C_{MAX} ($ng \cdot mL^{-1}$)	T_{MAX} (hr)	AUC/dose ($ng \cdot hr \cdot mL^{-1} \cdot mg^{-1}$)	C_{MAX}/dose ($ng \cdot mL^{-1} \cdot mg^{-1}$)
				Metabolite 1		15,400	322	3 min	770	16.1
				Metabolite 2		19,300	643	3 min	965	32.2
	Male Sprague-Dawley rats	Liquiritigenin, oral		Liquiritigenin	50	297	26.0	7 min	5.94	0.52
				Metabolite 1		34,833	132.8	15 min	697	2.7
				Metabolite 2		32,000	246.7	15 min	640	4.9
	STZ-induced diabetic rats			Liquiritigenin		443	49.7	5 min	8.86	1.0
				Metabolite 1		32,333	298	15 min	647	6.0
				Metabolite 2		33,000	558	15 min	660	11.2
Hasegawa, 2010 (76)	Male Donryu rats	Morphine	∞	Morphine	15	12,200	–	–	813	–
	STZ-induced diabetic rats					7,000	–	–	466	–

The flavone glycoside baicalin, found in the Chinese herb *Scutellaria baicalensis*, was reported to be excreted at faster rates in diabetic compared to healthy rats (Liu et al., 2009). Though diabetic rats had significantly higher plasma AUC of the compound when provided a 200 mg/kg baicalin i.g. dose (48.48 vs. 100.77 $\mu g \cdot hr \cdot mL^{-1}$; normal vs. diabetic), the normal group had significantly higher levels of the compound when given a 12 mg/kg dose by i.v. (18.02 vs. 11.20 $\mu g \cdot hr \cdot mL^{-1}$; normal vs. diabetic). Since baicalin is transported across the ileum after the glucuronide moiety is cleaved, both groups of rats were tested for activity of β-glucuronidase, the enzyme that catalyzes the cleavage. There was a 1.7-fold increase in activity of this enzyme in the duodenum of the diabetic animals, demonstrating a mechanism for the observed apparent increased bioavailability via the oral dose. Still unanswered in this study is the underlying explanation for increased systemic clearance that drives the decreased baicalin plasma concentrations in the diabetic rats compared to the normal animals when given the i.v. dose.

Cao *et al.* (Cao et al., 2012) reported that plasma clearance and/or decreased absorption of salicylic acid was altered in Goto-Kakizaki (GK) diabetic rats, a model for a non-obese diabetic phenotype, as compared to normal Wistar rats. Salicylic acid was administered in feed, and plasma samples were collected over the course of 15 weeks. Concentration of salicylic acid in GK rat plasma was consistently significantly lower compared to the Wistar rats over the course of the test period, which may be due to increased apparent clearance in GK (94.6 $mL \cdot h^{-1}kg^{-1}$) compared to Wistar rats (68.0 $mL \cdot h^{-1}kg^{-1}$). Since this study administered the salicylic acid in their feed, it is also possible

that there were alterations in digestion or intestinal transport that decreased absorption of this compound. Still, the authors noted the clinical implications of the lower salicylic acid plasma values in the diabetic rats by concluding that diabetics may need increased dosage of compound to overcome increased excretion rates (Cao et al., 2012).

Similar effects have been observed in non-phenolic compounds that rely on similar transport and metabolizing pathways. Yu *et al.* (Yu et al., 2010) noted elevated plasma clearance of the phenolic-like alkaloid jatrorrhizine in diabetic Sprague-Dawley rats compared to healthy controls. In this study, a plant extract rich in jatrorrhizine was administered by intestinal perfusion, and plasma from the portal vein was collected. The results showed that the C_{max} (maximum concentration) in diabetic animals was 9.5-fold greater and the AUC was 7.5-fold greater than the non-diabetic control. Probing P-gp function with Rho123 found it to be less active in the diabetic rats, and Western blot confirmed that there was also decreased protein levels of P-gp in all segments of the small intestine. The authors commented that compared to the normal group, there was a greater increase in the portal vein concentration of jatrorrhizine in the diabetic animals, but not a corresponding higher amount of compound in the peripheral plasma. This first appears to be a paradox since the compound must be transported to another compartment after going through the portal vein, but they seem to support the data from Liu *et al.* suggesting increased clearance from systemic circulation to urine in diabetic animals due to greater transport of jatrorrhizine across the small intestine but lower levels in the periphery.

1.9 Human Clinical Trials suggest Obesity and Diabetes affect Xenobiotic Transport and Metabolism

Since bioactivity of phenolics is largely dependent upon their metabolism by key Phase II enzymes (Silberberg et al., 2006), it is necessary to consider the systems that may be altered in diseased conditions. Early research documented fundamental differences in the xenobiotic transport and metabolizing systems in obese or diabetic individuals (Gwilt et al., 1991). While many studies in this area have focused on pharmaceuticals, interestingly, the effect of disease states on these systems has also been investigated using phenolic compounds. As both phenolics and many pharmaceuticals target the Phase II and III metabolizing systems, research utilizing pharmaceuticals is also included in this discussion.

For instance, Abernethy *et al.* demonstrated the potential influence of obesity on xenobiotic metabolizing systems (Abernethy et al., 1983). In this study, normal weight and obese subjects were given a 30 mg oral dose of the benzodiazepine oxazepam, excreted after glucuronidation, and it was found that plasma clearance of oxazepam increased significantly in the obese (157 mL/min) compared to the normal weight group (50 mL/min). Although doses for many pharmaceutical treatments reflect changes in dose based on weight, it is possible that obesity can fundamentally increase levels or activity of Phase II metabolizing enzymes (Brill et al., 2012). This elevated metabolism can result in an increase in the rate at which phenolic compounds are metabolized and excreted and may, in turn, decrease the potential for effective biological activity.

In addition, clinical studies show that pharmacokinetic parameters of the stilbenoid *trans*-resveratrol differ depending on health status of the population (summarized in **Table 2**). Boocock *et al.* (Boocock et al., 2007) administered 1,000 mg of *trans*-resveratrol to healthy, non-obese participants and observed an AUC of 544.8 $ng \cdot hr \cdot mL^{-1}$ and C_{max} of 117 ng/mL, but when 1,500 mg of *trans*-resveratrol was administered to an overweight/obese (mean BMI = 31.8) population with hepatic steatosis, it resulted in an AUC of 705 $ng \cdot hr \cdot mL^{-1}$ and a C_{max} of 65.7 ng/mL (Chachay et al., 2014). These studies can be more easily compared when normalizing these parameters by dose, which reveals that the healthy participants in Boocock *et al.* had an approximately 15% greater AUC/dose and a more striking 167% greater *trans*-resveratrol C_{max}/dose compared to the overweight/obese population in Chachay *et al.*, demonstrating a potentially reduced ability to achieve a threshold for therapeutic concentrations. As non-alcoholic hepatic steatosis has been reported to be present in approximately 66% of obese (Sabir et al., 2001) and even 18% of lean populations (Younossi et al., 2012), early stages of this condition may be playing a role in liver function. Thus, proper liver function may potentially be affecting phenolic metabolism in certain studies given prevalence of hepatic steatosis in the population.

Table 2. Comparison of clinical pharmacokinetic studies using resveratrol in overweight/obese and normal weight populations.

Study and Reference	Health Status	Source	Time (hr)	Compound	Dose ($mg \cdot kg^{-1}$)	AUC ($ng \cdot hr \cdot mL^{-1}$)	C_{MAX} ($ng \cdot mL^{-1}$)	T_{MAX} (hr)	AUC/dose ($ng \cdot hr \cdot mL^{-1} \cdot mg^{-1}$)	C_{MAX}/dose ($ng \cdot mL^{-1} \cdot mg^{-1}$)
Chachay, 2014 (72)	Overweight/obese (mean BMI = 31.8) humans with hepatic steatosis	*trans*-resveratrol capsule	6	*trans*-resveratrol (no metabolites measured)	1,500	705	65.7	1	0.47	0.0438
Boocock, 2007 (71)	Healthy humans	*trans*-resveratrol capsule	24	*trans*-resveratrol	1,000	544.8	117	0.76	0.54	0.117
				Glucuronide 1		3,059	473.6	2.25	3.06	0.475
				Glucuronide 2		2,589	672.6	1.75	2.59	0.673
				Sulfate		10,053	2,102	2	10.1	2.10
				Resveratrol + metabolites		16,246	3,365	–	16.3	3.37

1.10 Pre-clinical and In Vitro Data Suggest Obesity and Diabetes Affect the Phase II and III Metabolizing Systems

The observed alterations in xenobiotic metabolism in obese and diabetic humans coincide with animal research done by Liu *et al*. (Liu et al., 2012) and additional studies which show differential expression of key Phase II metabolizing enzymes according to health status (see **Table 3**).When male Wistar diabetic rats were orally given 400 mg/kg of the xanthone mangiferin (a C-glycoside found in plants in the Anacardiaceae family), plasma AUC was significantly greater in the diabetic rat group (3,817.5 vs. 8,953.4 $ng \cdot hr \cdot mL^{-1}$; healthy vs. diabetic). Although PK parameters of metabolites were not determined, liver mRNA expression of select *Ugt1a* family enzymes increased 2.28-3.93-fold, *Ugt2b8* increased 4.39-fold, and *Sult1a1* increased 1.62-fold in diabetic rats. In contrast, the liver mRNA expression of *Comt* decreased 1.72-fold, *Ugt2b*-family enzymes decreased by approximately 2-fold, and there was a 6.67-fold reduction in *Sult1c1*. Overall, these data indicate that differences in pharmacokinetic parameters in the diabetic state may be due to fundamental alterations in metabolizing systems.

Table 3. Summary of pre-clinical molecular-based studies.

Study and Reference	Model and Health Status	Tissue Type	Xenobiotic, Transport, and Metabolizing System	Direction of Change
Wang, 2002 (76)	Male Wistar STZ-induced diabetic rats	Liver	COMT activity	↓
Braun, 1998 (85)	Male B/Wor diabetic rats	Liver	*Ugt1a1* mRNA; protein; activity	↑
Burant, 1994 (105)	Male Sprague-Dawley STZ-induced diabetic rats	Enterocytes	*Glut2*, *Glut5*, *Sglt1* mRNA and protein	↑
Maeng, 2007 (111)	Male Sprague-Dawley STZ-induced diabetic rats	Brain	P-gp protein	↑
Zhang, 2011 (89)	Male Sprague-Dawley STZ-induced diabetic rats	Liver	*Abcb1a*, *Abcb1b* mRNA; protein (P-gp)	↓ / no change
		Intestinal mucosa	*Abcb1a*, *Abcb1b* mRNA and protein (P-gp)	↓
		Kidney	*Abcb1a* mRNA; *Abcb1b* mRNA; protein (P-gp)	↑ / no change / ↑
Liu, 2006 and 2007 (91,92)	Male Sprague-Dawley and ICR STZ-induced diabetic rats	Brain cortex	P-gp protein	↓
Nawa, 2010 (90)	Male ddY STZ-induced diabetic mice	Ileum	P-gp protein	↓
van Waarde, 2002 (93)	Male Wistar STZ-induced diabetic rats	Liver	*Mdr2* mRNA and protein	↑
		Liver	*Mrp2* mRNA; protein	No change / ↓
Nikooie, 2013 (94)	Male Wistar STZ-induced diabetic rats	Soleus muscle	*Mct1* mRNA and protein	↓
Yu, 2010 (66)	Male Sprague-Dawley STZ-induced diabetic rats	Small intestine	P-gp protein	↓

Table 3, con'd. Summary of pre-clinical molecular-based studies.

Study and Reference	Model and Health Status	Tissue Type	Xenobiotic, Transport, and Metabolizing System	Direction of Change
Liu, 2012 (75)	Male Wistar STZ-induced diabetic rats	Duodenum and jejunum	*Mdr1a* and *Mdr1b* (P-gp) mRNA	↓
		Ileum	*Mdr1a* and *Mdr1b* (P-gp) mRNA	↑
		Liver	*Comt* mRNA	↓
		Liver	*Ugt1a3*, *Ugt1a7*, *Ugt2b3*, *Ugt2b6*, *Ugt2b12* mRNA	↑
		Liver	*Ugt2b3* and *Ugt2b8* mRNA	↓
		Liver	Sult1b1 mRNA	↓
Kang, 2010 (86)	Male Sprague-Dawley STZ-induced diabetic rats	Liver	UDP-glucuronic acid	↑
		Intestine	UDP-glucuronic acid	no change
Hasegawa, 2010 (83)	Male Donryu STZ-induced diabetic rats	Glucuronidation probed using morphine	UGT	↑
		Liver	*Ugt2b1* mRNA	↑
		Liver	*Mrp2* mRNA; *Mrp3* mRNA	↓ / ↑
Kim, 2004 (87)	Male Sprague-Dawley vs. obese Zucker rats	Liver	*Ugt1a1*, Ugt*1a6*, Ugt*2b1*, *Mrp2*, *Oatp2* mRNA	↓

Table 3, con'd. Summary of pre-clinical molecular-based studies.

Study and Reference	Model and Health Status	Tissue Type	Xenobiotic, Transport, and Metabolizing System	Direction of Change
Xu, 2012 (84)	Male obese C57BL/6J $Lep^{ob/ob}$ mice	Liver	*Ugt1a1*, *Ugt1a6*, *Ugt1a9*, *Ugt2a3*, *Ugt3a1*, *Ugt3a2* mRNA	↑
		Liver	*Ugt2b1* mRNA	↓
Lucas-Teixeira, 2002 (77)	Obese Zucker rats	Jejunal mucosa	COMT activity	↑
Koide, 2011 (78)	Male C57BL/6J diet-induced obese rats	Liver	Total UGT activity	↑
		Liver	SULT2A1 protein and activity	↓
Kim, 2004 (87)	Female C57BL/6 mice treated with LPS	Liver	*Sult2a1* mRNA and total SULT activity	↓
Shimada, 1999 (81)	Male Sprague-Dawley rats treated with LPS	Liver	Total SULT activity	↓
Ho, 2007 (113)	Male Sprague-Dawley rats treated with LPS	Liver	*Mdr1b* mRNA	↑

Further, Wang *et al.* reported that COMT methylation activity measured over 30 min decreased by 28.1% in the liver of male Wistar rats with uncontrolled diabetes, but the enzyme's function was rescued by restoring blood glucose levels to a normal range (Wang et al., 2002). This suggests that the downstream effects from chronic elevated blood glucose may contribute to alterations in methylation activity. In contrast, COMT V_{max} increased by 80.5% in obese Zucker rats in the jejunum compared to lean rats (Lucas-Teixeira et al., 2002). Thus, changes in COMT activity and expression appear to be dependent on tissue type and homeostatic aberrations, such as elevated blood glucose levels associated with diabetes.

Protein levels of SULT enzymes, along with their activity, have also been reported to be affected by obesity, diabetes, and associated comorbidities. For instance, male C57BL/6J mice with diet-induced obesity have decreased protein levels of SULT2A1 in liver (Koide et al., 2011). Additionally, research on human clinical samples has shown similar trends (see summary in **Table 4**). Both *SULT1C4* mRNA and protein expression has been reported to increase in humans with fatty liver, a common comorbidity of obesity (Hardwick et al., 2013), however liver SULT1A1 protein levels are significantly lower in individuals with either liver steatosis or diabetes (Yalcin et al., 2013). Thus, it appears that specific isoforms are differentially impacted through pathways mediated by inflammatory signaling associated with obesity and diabetes. For example, liver *Sult* isoforms *1a1*, *1b1*, and *1c1* mRNA expression and their respective proteins decreased in male rats after administering the inflammatory agent bacterial lipopolysaccharide (LPS) (Shimada et al., 1999). However, there was no reduction in *Sult* mRNA expression in rats

pretreated with the anti-inflammatory compound dexamethasone, which coinsides with results from another study in female C57BL/6J mice that showed a decrease in SULT activity under inflammatory conditions (Kim et al., 2004a). As such, both obesity and diabetes, as well as related inflammatory stress from these conditions appear to have a role in ultimate status of SULT enzymes and their activities.

Data from animal and human studies provide conflicting information on the impact of obesity and/or diabetes on glucuronidation processes. Male diabetic Donryu rats had approximately twice the circulating concentration of morphine-3-glucuronide from 185-365 min after an initial 15 mg/kg morphine IV dose compared to normal controls (Hasegawa et al., 2010). Additionally, Xu *et al*. (Xu et al., 2012) found that obese Lep$^{ob/ob}$ compared to normal C57BL/6J mice had significantly increased acetaminophen glucuronidation velocity in hepatocytes (0.15 vs. 0.23 nmol·min^{-1}mg^{-1}·protein), which correlated to an approximately 5-fold increase in hepatocyte mRNA expression of *Ugt1a1* and *Ugt3a1* isoforms in the obese mice. Comparing male diabetic BB/Wor to normal Wistar rats showed that UGT1A1 enzyme activity increased from 0.231 to 1.816 nmol·min^{-1}·mg^{-1} protein in permeabilized liver microsomes (Braun et al., 1998). In streptozocin-induced diabetic male Sprague-Dawley rats, plasma AUC licorice-derived flavanone metabolite liquiritigenin-7-*O*-glucuronide increased from 738 to 1,160 µg·min·mL^{-1} and C_{max} from 24.6 to 38.6 µg/mL compared to normal controls (Kang et al., 2010). In contrast, obese Zucker rats have a statistically significant 33-63% decrease in the hepatic mRNA expression of the *Ugt* isoforms*1a1*, *1a6*, and *2b1* compared to lean Sprague-Dawley rats (Kim et al., 2004b). In humans, the diabetic condition has been

reported to decrease both glucuronide enzyme activity and mRNA expression of the UGT2B7 isoform of the enzyme by approximately 50% (Dostalek et al., 2011). However, obese individuals are reported to have increased concentrations of oxazepam glucuronide metabolites (Abernethy et al., 1983), and mRNA expression of *UGT1A9* and *2B10* isoforms has been shown to be significantly higher in humans with non-alcoholic fatty liver disease (Hardwick et al., 2013). While data overall are somewhat mixed, evidence does support alteration in glucuronidation processes due to the obese or diabetic state. Since these changes in glucuronidation can affect overall metabolite profiles, excretion of phenolic compounds, and by extension apparent C_{max} values, they may potentially impact reaching threshold concentrations to elicit a physiological response.

Table 4. Summary of human molecular-based studies.

Study and Reference	Population and Health Status	Tissue Type	Xenobiotic, Transport, and Metabolizing System	Direction of Change
Hardwick, 2013 (79)	Non-alcoholic fatty liver disease	Liver	*UGT1A9* and *2B10* mRNA; protein; total UGT activity	↑ / no change / no change
			SULTA1C4 mRNA; protein; total SULT activity	↑ / ↑ / ↓
Yalcin, 2013 (80)	Liver steatosis	Liver	*SULT1A1* mRNA; protein; activity	no change / ↓ / ↓
		Liver	*SULT1A3* mRNA; protein; activity	no change / ↑ / ↓
Metz, 2008 (97)	Male and female, lean (mean BMI = 22.6) vs. obese (mean BMI = 35.3)	Leg skeletal muscle	MCT1 protein	no change
Dostalek, 2011 (88)	Male and female diabetics	Liver	*UGT1A9, UGT1A1, and UGT2B7* mRNA	↓
		Kidney	*UGT1A9, UGT1A1, and UGT2B7* mRNA	↓
Yalcin, 2013 (80)	Diabetics	Liver	*SULT1A1* mRNA; protein; activity	no change / ↓ / ↓
		Liver	*SULT1A3* mRNA; protein; activity	no change / no change / ↓
Juel, 2004 (96)	Male Type II Diabetics	Leg skeletal muscle	MCT1 protein	↓
Kim, 2004 (87)	Hep 3B cells treated with TNF-α and IL-1	Hepatocytes	*SULT2A1* mRNA	↓

Disease state may also impact the Phase III metabolizing system by affecting P-gp function and expression levels. Comparing 8-week old male Sprague-Dawley rats to diabetic rats treated with insulin shows significantly greater Rho123 concentrations in the liver (69.4 vs. 120.9 ng/g; normal vs. diabetic), intestinal mucosa (101,135 vs. 210,123 ng/g; normal vs. diabetic), and kidney (422.5 vs. 578.0 ng/g; normal vs. diabetic) of diabetic rats demonstrating impaired P-gp function (Zhang et al., 2011). In ileal tissue of diabetic male ddY mice, P-gp protein levels were 60% that of the control (Nawa et al., 2010). Decreased P-gp function has also been demonstrated in the brain of diabetic male Sprague-Dawley rats by showing that the ratio of brain tissue concentration to plasma level of the P-gp substrate VCR is significantly greater in the diabetic group as compared to the control (0.023 vs 0.072 mL/brain; normal vs. diabetic) (Liu et al., 2006). This has been confirmed in male diabetic ICR mice, with brain concentrations of Rho significantly greater in diabetic rats (13.69 ng/g) compared to normal controls (10.24 ng/g) (Liu et al., 2007). In contrast, van Waarde *et al.* (van Waarde et al., 2002) interestingly found that the liver of diabetic Wistar rats had a 530% increase in protein expression of MDR2 and a corresponding elevation in bile salt secretion compared to control.

Though efflux transporters have an important role in the excretion of phenolics, expression of transporters that drive transport of phenolics into the enterocyte and other cells may also be altered in obesity and diabetes. In diabetic male Wistar rats, *Mct1* mRNA expression in soleus muscle is significantly lower than control (Nikooie et al., 2013), in addition to decreased expression of MCT1 protein in the plantaris muscle

(Enoki et al., 2003). Though this pattern has also been reported in male diabetic patients with decreased skeletal muscle expression of MCT1 (Juel et al., 2004), obese individuals did not show differences in muscle MCT1 protein levels compared to control (Metz et al., 2008). Though MCT1 in human hepatic or intestinal tissue was not directly measured in these studies, these may similarly be affected in diseased conditions.

1.11 Additional Potential Factors from the Obese and Diabetic Condition May Affect Phenolic Pharmacokinetics

Although this review focused on critical xenobiotic metabolizing and transport systems altered by obesity and diabetes, there are additional factors to consider that may influence phenolic pharmacokinetic parameters in these conditions. One such example is that there is an increase in glomerular filtration rate from the diabetic condition (Premaratne et al., 2015), which could decrease plasma phenolic concentrations by increasing their elimination through urine. In addition, kidney disease that may result from late-stage diabetes can alter factors that influence xenobiotic metabolism (Nolin et al., 2008).

Differences in the gut function between diabetic and normal rats may also partially explain differential absorption of pharmaceutical compounds. Ogata *et al.* (Ogata et al., 1997) compared absorption of the peptide cyclosporin A between GK and Wistar rats by administering the compound orally or by i.v. When 10 mg/kg cyclosporin A was given by i.v., the diabetic and normal group had similar plasma AUC of the compound (21.9 vs. 20.6 $\mu g \cdot hr \cdot mL^{-1}$; normal vs. diabetic), but when orally administered,

the diabetic rats had significantly lower plasma AUC concentrations (11.3 vs. 1.8 $\mu g \cdot hr \cdot mL^{-1}$; normal vs. diabetic). Since this compound is absorbed in the small intestine, gastric emptying was determined using radiolabeled sodium chromate. While 90% of the marker could be recovered in the distal section of the ilium of the normal group after 2 h, only 60% could be recovered in the diabetic rats with 25% of the marker still remaining in the stomach. It is important to point out that while this study compared only two different breeds of rats, these results may be applicable in a broader sense to the absorption of phenolic compounds because gut transit time can impact PK parameters.

Another possible factor to consider is that of intestinal microbiota since microbiota profiles can change under diabetic or obese conditions (Forslund et al., 2015; Ley et al., 2005). As phenolics have low first-pass bioavailability, they reach the lower gut where they may be catabolized into low-molecular weight compounds that are potentially absorbed and then enter systemic circulation where they can exert biological activity (Del Rio et al., 2013). Due to beneficial effects of phenolics potentially being mediated by gut microbiota, disease states that perturb microbiota composition may alter their efficacy (Tomás-Barberán et al., 2014). For example, Selma *et al.* (Selma et al., 2015) found that production of bioactive catabolites of ellagic acid (urolithins) differ between overweight/obese and normal weight individuals, decreasing potential efficacy derived from these compounds.

In the diabetic condition, there are alterations to intestinal tissues that occur due to elevated blood glucose levels, which may also affect phenolic transport. These

changes may emerge because expression of the small intestinal carbohydrate transporters SGLT1 and GLUT2 increase in diabetic rats, though controlling blood glucose with proper insulin treatment restores them to normal levels (Burant et al., 1994). This alteration can then potentially increase uptake of certain phenolics whose transport is associated with glucose transporters.

1.12 Potential Underlying Mechanism for Altered Xenobiotic Metabolizing and Transporting Systems in Obese and Diabetic Populations

A potential underlying mechanism for the differences in xenobiotic transport and metabolism observed in obese and diabetic populations involves chronic, low-grade inflammatory response prevalent in these conditions (Donath and Shoelson, 2011; Neels and Olefsky, 2006). In a study mentioned previously, administering LPS to Sprague-Dawley rats resulted in a 36.8% reduction in hepatic sulfonation activity (Shimada et al., 1999), which aligns with another report that LPS-induced secretion of inflammatory cytokines and stimulation of the inflammatory processes alters drug metabolism (Waring et al., 2013). Similarly, elevated insulin levels from diabetes may also induce alterations that affect Phase II enzymes (Kim and Novak, 2007). In addition, Thibault *et al.* (Thibault et al., 2007) reported that individuals with inflammatory bowel disease have decreased expression of the MCT1 transporter, which was replicated in a mechanistic *in vitro* study demonstrating that treating the intestinal cell line HT-29 with the inflammatory cytokines IFN-γ and TNF-α dose dependably decreases mRNA expression and protein levels of this transporter. The authors of the study hypothesize

that the mechanism underlying this phenomenon may be due to an inflammatory response element in the promoter region of the *MCT1* gene. Hence, inflammatory processes mediated though NF-κB signaling or another inflammatory signaling pathway likely play a role in expression of MCT1 and by extension may impact absorption of certain phenolics.

Further, the inflammatory response mediated through NF-κB induction is known to modulate the expression of P-gp (Maeng et al., 2007). A review on P-gp expression in the intestine suggests that it is increased in diabetic humans due to elevated inflammatory cytokines and blood glucose (Kobori et al., 2013). In addition, the transporter MRP3 responds to inflammatory stimuli, and has been shown to become either up or downregulated depending on the animal model used in the study (Ho and Piquette-Miller, 2007). More research is needed in humans to provide a definitive answer on how disease states affect these transporters, however existing data do suggest that the diabetic condition likely increases efflux transporter activity that could then potentially increase excretion of phenolics.

Additional evidence supports the relationship between increased inflammatory status and altered xenobiotic metabolizing and transporting systems. Individuals with Crohn's disease, a condition of elevated inflammation in the gut, have increased expression of P-gp in intestinal and hepatic tissue (Fakhoury et al., 2006) and increased permeability of tight junctions, making the gut less effective in maintaining barrier ability (Al-Sadi et al., 2008). In addition, the elevation of protein kinase C in the diabetic state leads to an increase in the p65 subunit of the NF-κB transcription factor, which

then, in turn, increases P-gp levels in the liver (Kameyama et al., 2008; Maeng et al., 2007). Also, MRP2/3 have both been found to be altered by increased inflammatory status (Ho and Piquette-Miller, 2007; Le Vee et al., 2009). **Figure 4** synthesizes this information in a potential model to explain the altered absorption of phenolic compounds apparent in those with increased inflammatory status due to obesity and diabetes.

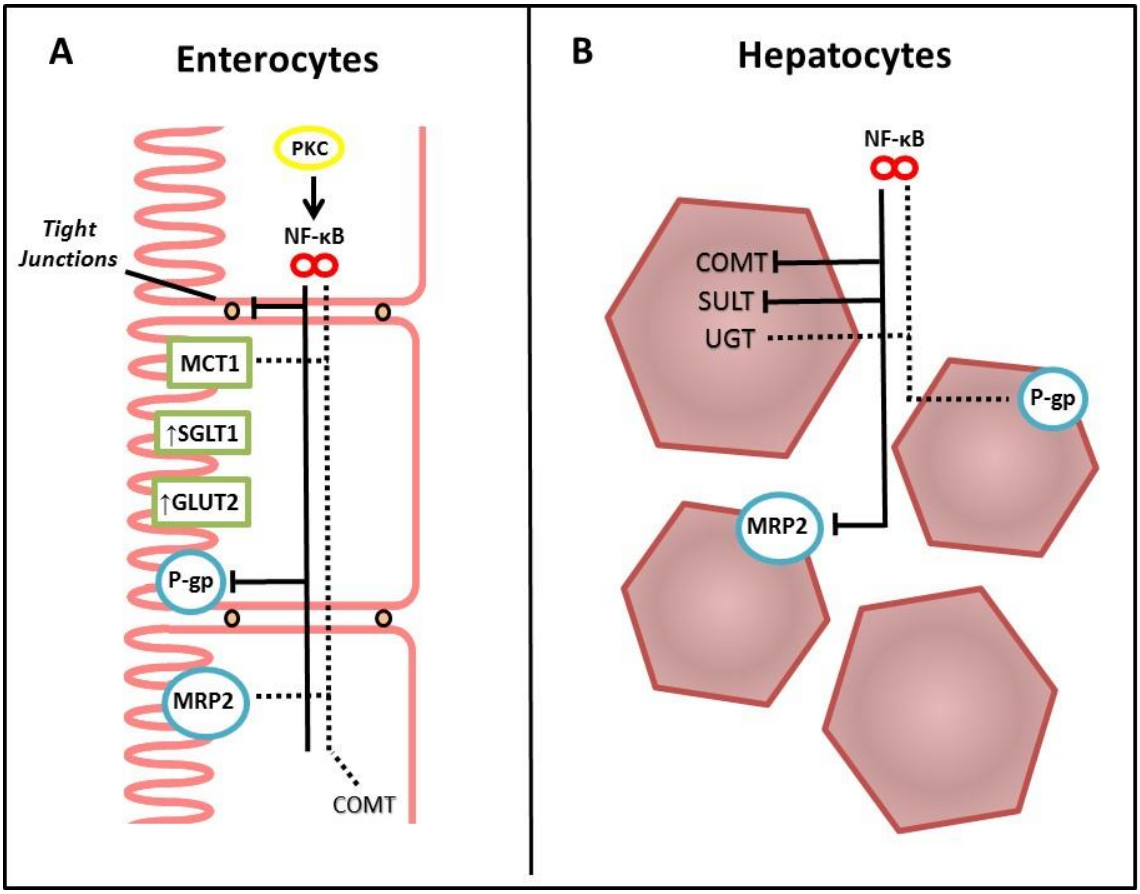

Figure 4. Potential mechanism for altered xenobiotic transport and metabolizing systems in human (A) intestinal and (B) hepatic tissues. Increased NF-κB mediated inflammation resulting from the diabetic and obese condition are believed to be involved in the alterations. Dashed lines indicate potentially affected by increased NF-κB signaling, and solid line indicates negatively affected.

1.13 CONCLUSIONS & IMPLICATIONS

Absorption, metabolism, and transport of phenolic compounds to target tissues are all essential for effective delivery of their bioactivity from food and dietary supplements, and these processes appear to be affected by disease states such as obesity or diabetes. The overall effect from these diseases is that they may create a situation where application of phenolic-rich foods or supplements may not be as effective as expected due to impairment of absorption and metabolism of phenolic compounds and increased excretion. Subsequently, formulations and therapies may have to be adjusted in order to accommodate for altered production of phenolic metabolites into circulation and target tissues. While some evidence does exist for the possible sources of the differences, it is critical to fully understand the underlying mechanism for the observed differences in phenolic effectiveness and/or bioavailability and metabolism between healthy and obese/diabetic individuals. Only then will strategies to enhance effectiveness of preventative ameliorative therapies utilizing these phenolic can be improved.

Continued research on the fundamentals of the absorption and metabolism of phenolic compounds can help inform future studies utilizing phenolics in individuals with obesity or diabetes. One goal of future research should be to specifically compare the differential absorption of phenolic compounds in diseased versus healthy individuals. Study design will have to be carefully addressed in order to elucidate the mechanism for decreased absorption of phenolic compounds in those with obesity or diabetes. Including administration of phenolic compounds by i.v. would allow for assessment of

clearance from plasma and oral administration can assess absorption across the gut epithelium. Experiments of this nature are needed to confirm differences in phenolic absorption parameters between normal and diseased individuals. With evolving research that targets the fundamentals of phenolic absorption comparing healthy and diseased populations, researchers and product developers will be able to leverage these findings to develop products and strategies that better target obese or diabetic populations.

CHAPTER 2. CHARACTERIZATION AND QUALITATIVE ANALYSIS OF PLASMA-TARGETED METABOLITES OF CATECHIN AND EPICATECHIN

As part of the manuscript "Synthesis and quantitative analysis of plasma-targeted metabolites of catechin and epicatechin," JW Blount[1], BW Redan[2], MG Ferruzzi[2,3], BL Reuhs[3], BR Cooper[4], JS Harwood[5], V Shulaev[1], G Pasinetti[6], RA Dixon[1], published in J. Agric. Food Chem. 2015, 4;63(8):2233-40. [1]Department of Biological Science, University of North Texas, [2]Department of Nutrition Science, Purdue University, [3]Department of Food Science, Purdue University, [4]Bindley Bioscience Center, Purdue University, [5]Purdue University Interdepartmental NMR Facility, [6]Department of Psychiatry, The Mount Sinai School of Medicine

As this publication was a collaboration, the present chapter focuses on the characterization and qualitative analysis of the metabolites since our collaborators performed their synthesis.

2.1 Introduction

Plant-derived polyphenolic compounds possess diverse biological activities, including strong anti-tumor, anti-oxidant, anti-inflammatory, and anti-microbial

activities (Dixon, 2001; Guo et al., 2009; Ishikawa et al., 1997). Previous work from our laboratories has shown that polyphenolic compounds from multiple sources, including a specific grape seed polyphenolic extract (GSPE), are capable of improving cognitive functions and reducing brain neuropathology in animal models of Alzheimer's Disease through multiple mechanisms (Wang et al., 2008, 2012). At least a part of this effect may be mediated through interference with aggregation of β-amyloid peptides into neurotoxic, soluble high-molecular weight species (Wang et al., 2008).

GSPE is a complex mixture of proanthocyanidins (PACs, both oligomeric and polymeric) and their monomeric units consisting of the flavan-3-ols (+)-catechin (C) and (-)-epicatechin (EC) (Sharma et al., 2011). The flavan-ol units are assembled into the various types of oligomers and polymers through C4→C8 or C4→C6 interflavan bond and the individual units in the polymer can also be substituted with gallic acid. The monomeric units, rather than the oligomers or polymers, are bioavailable and are the bioactive components in the GSPE (Wang et al., 2012). These bioactive components, in the form of glucuronidated and/or methylated phase II metabolites, reach the brain at a concentration of 300-400 nM after 10 days of repeated dosing (Wang et al., 2012). While knowledge of all metabolites found in plasma may be of broader significance for understanding the therapeutic effects of GSPE on a range of disease phenotypes, mechanistic studies, require knowledge of the exact structures of metabolites that accumulate in brain or other tissue. To initiate such studies, we implemented a semi-synthetic approach to generate sufficient quantities of flavan-3-ol metabolites for unequivocal identification. We demonstrated that recombinant human

glucuronosyltransferases of the UGT1A and UGT2B families can efficiently glucuronidate epicatechin or 3'-*O*-methyl epicatechin in vitro, suggesting a method for the semi-synthesis of brain-targeted flavan-3-ol metabolites (Blount et al., 2012). Of twelve enzymes tested, UGT1A9 was the most efficient, and this enabled us to generate 3'-*O*-methyl-epicatechin-5-*O*-glucuronide to 50% overall yield (Blount et al., 2012). In the present study, we have extended the above approach, in combination with chemical methylation of catechin and epicatechin with iodomethane, to generate authentic standards of the catechin and epicatechin metabolites found in the plasma and brains of rats treated with GSPE. The structures of the metabolites were rigorously identified, and we present a robust, validated protocol for the measurement of the levels of these compounds in animal fluids.

2.2 Materials and Methods

2.2.1 Solid Phase Extraction (SPE) of Catechin and Epicatechin Metabolites from Rodent Plasma

Rodent plasma samples were obtained from a previous pharmacokinetic study using GSPE (Wang et al., 2012) that received approval from the Animal Care and Use Committee at Purdue University. Plasma aliquots from control animals (no GSPE) and treated animals (with GSPE) that was collected at sacrifice were thawed from -80°C storage and quickly pooled separately at Purdue University. These control and GSPE plasma samples were then re-aliquoted into individual vials and re-frozen at -80 °C until analysis. For target assessment purposes, typical plasma was diluted fivefold with blank

plasma to produce low dose plasma. Blank, low, and normal plasma samples were then used for analytical validation at Purdue University and a parallel set of samples was sent to University of North Texas for cross-lab validation.

Samples of frozen plasma were thawed at room temperature, and 300 µL of acidified saline (9g NaCl in 1000 mL 0.1% v/v formic acid/H_2O) were added to 200 µL of plasma with vortex mixing. A SPE cartridge, 30 mg Oasis HLB column (Waters Co, Milford, MA), was activated by addition of 1 mL MeOH followed by 1 mL DI H_2O, after which the plasma/saline mixture was loaded. Loaded SPE column was first washed with 1 mL 1.5 M formic acid, followed by 1 mL 95:5 v/v H_2O: MeOH, and the eluate discarded. Metabolites were then eluted with 2 mL 0.1% v/v formic acid/MeOH. Following elution, ethyl gallate (10 µL of 10 µM stock solution) was added to the eluates as the internal standard prior to samples being dried under vacuum (<50 mm Hg) at 37°C. The residues were re-suspended in 160 µL 0.1% v/v formic acid/H_2O + 40 µL 0.1% v/v formic acid/acetonitrile, sonicated for 10s, vortex mixed for 10s, then transferred to centrifuge tubes, which were centrifuged at 18,000*g* for 5 min to remove any particulates. The clear supernatants were carefully transferred to 300 µL HPLC vials for analysis.

The ability of this extraction method to account for potential matrix effects were assessed by comparing response of individual metabolite standards in the presence or absence of blank plasma extracted matrix. Briefly, standards of catechin and its major metabolites in GSPE rodent plasma at 50, 100, and 500 nM were resuspended either in pure solvent (160µL 0.1% v/v formic acid/H_2O + 40 µL 0.1% v/v formic acid/acetonitrile - Standard) or in resolubilized blank plasma extracted by SPE (Standard + Matrix). By

comparison of response between standards injected with or without plasma matrix an estimate of matrix effects was established by using the following equation: % matrix effect (% ME) = (peak area of standard with matrix – peak area of pure standard)/(peak area of pure standard) x 100% (Ćirić et al., 2012).

2.2.2 LC/MS Analysis of C/EC Metabolites Extracted from Rodent Plasma

2.2.2.1 LC-TOF-MS for unknown metabolite characterization

Separation was achieved on an Agilent 1100 HPLC system using a Waters XBridge BEH shield RP-C18 XP 2.1 x 100 mm 2.5 µm column following a 2 µm frit filter. The column and autosampler were maintained at 40 °C and 10 °C, respectively. Mobile phase A was 0.1% formic acid/H_2O and B was 0.1% formic acid/ACN. A linear gradient for elution was used at a constant flow rate of 0.26 mL/min. Initial conditions 5% B; 0-12 min: 5-15% B; 12-18min: hold at 15% B; 18-24 min: 15-40% B; 24-30 min: 40-50% B; 30-31 min: 50-5% B; 31-36 min: hold at 5% B to reset gradient. Injection volume was set to 10 µL.

Mass spectra of flavan-3-ol metabolites were obtained using an Agilent 6200 time-of-flight (TOF) mass spectrometer in negative mode with a mass range of 100-1000 m/z, acquisition rate of 1.4 spectra/s and 714.3 ms/spectra. Source parameters were as follows: gas temperature = 350 °C, drying gas = 9 L/min, nebulizer pressure = 35 psi, capillary potential = 3500 V, fragmentor voltage = 145 V, skimmer voltage = 60 V, and OCT 1 RF Vpp = 250 V.

2.2.2.2 LC-MS/MS for metabolite quantification

Separation was achieved on an Agilent 1200 HPLC system as described in the previous section, except for the gradient conditions as follows: initial conditions 10% B; 0-9 min: 10-35% B; 9-10 min: 35-65% B; 10-12 min: 65-10% B; 12-16 min: hold at 10% B to reset gradient.

Analytes were quantified using tandem mass spectrometry (MS/MS) with an Agilent 6460 triple quadrupole with electrospray ionization (ESI) in negative mode using selected reaction monitoring (SRM) mass transitions. Acquisition SRMs were: catechin/epicatechin, 289 → 245 m/z; catechin/epicatechin glucuronide, 465 → 289 m/z; methyl catechin/epicatechin glucuronide, 479 → 303 m/z; ethyl gallate, 197 → 169 m/z. All mass transitions used a dwell time of 200 ms and fragmentor voltage of 135 V. Source parameters were as follows: gas temperature = 350 °C, gas flow = 11 L/min, nebulizer pressure = 30 psi, sheath gas temperature = 350 °C, sheath gas flow = 11 L/min, capillary potential = 3500 V, and nozzle voltage = 1000 V.

Intraday repeatability was assessed by extracting and analyzing pooled plasma n = 5 times. Interday reproducibility was determined across n = 5 days using aliquot pooled plasma. Repeatability and reproducibility were determined as relative coefficient of variation (% CV) as calculated by (standard deviation/mean) x 100%. Limit of detection (LOD) was defined as S/N = 3 and limit of quantification (LOQ) as S/N = 5. Both values were obtained by producing serial dilutions of parent unmetabolized compounds and metabolites. Data are presented as mean ± standard deviation. Signal to noise (S/N) ratio was calculated using MassHunter software using the peak-to-peak method.

2.3 Results and Discussion

2.3.1 Flavan-3-ol metabolites in rat plasma after feeding fractionated GSPE

There are multiple compounds derived from the flavan-3-ols catechin (2,3-trans) and epicatechin (2,3-cis) found in mouse or rat plasma following the feeding of monomer enriched grape seed extract (Wang et al. 2012). LC-MS analysis has indicated that these compounds are glucuronides of (epi)catechin or methyl-(epi)-catechin. One of the most abundant metabolites in plasma was previously identified by micro NMR as 3'-*O*-methyl-epicatechin-5-*O*-β-D-glucuronide (**Figure 5**), and we developed a semi-synthetic route to this compound (Blount et al., 2012).

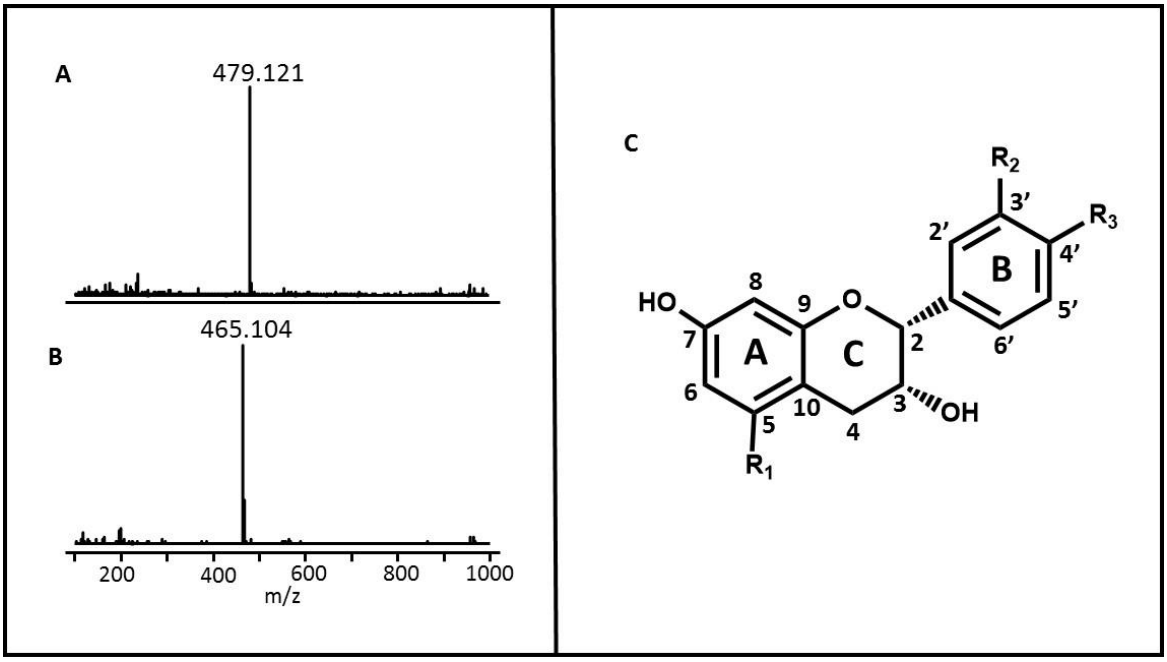

Figure 5. Flavan-3-ol derivatives in rodent plasma. (A, B) Representative mass spectra of flavan-3-ol metabolites in rodent plasma obtained on a LC/TOF system with a recording range of 100-1000 *m/z*. The mass spectra of (A) is consistent with the mass of methyl-C/EC glucuronide, while (B) is consistent with C/EC glucuronide. (C) Structure of 2,3-cis-flavan-3-ols. Epicatechin: $R_1 = R_2 = R_3 = OH$; 3'-O-methyl-epicatechin-5-*O*-β-D-glucuronide: R_1 = glucuronic acid; R_2 = OMe; R_3 = OH.

2.3.2 Methylation of catechin and epicatechin

Our strategy for generation of standards of the other (epi)catechin metabolites found in rat plasma was to: 1) synthesize methyl derivatives of catechin and epicatechin; 2) subject the (methylated) catechin and epicatechin to enzymatic glucuronidation; 3) compare LC-MS mobility and fragmentation pattern of the synthetic compound to those of the metabolites in plasma; 4) scale up the synthesis of compounds that match those in plasma and confirm their structures by NMR.

Optimization of reaction conditions, as outlined in Materials and Methods, resulted in maximum yields of 3'-*O*-methyl-(epi)catechin of around 20%. The

compounds corresponding to the 3'- and 4'-*O*-methyl isomers were purified by chromatography using a Biotage Isolera system.

2.3.3 Glucuronidation of (methyl)catechin and epicatechin, and matching of synthetic compounds to metabolites in plasma

Human recombinant glucuronosyltransferase UGT1A9, in the presence of UDP-glucuronic acid, converts epicatechin to a number of products, the major one being the 3'-*O*-glucuronide (a compound not found in the plasma of rats or mice fed GSPE), with minor amounts of the 5-*O*-glucuronide (a minor metabolite in the plasma) (Blount et al. 2011). 3'-*O*-methyl epicatechin is efficiently converted to 3'-*O*-methyl-epicatechin 5-*O*-glucuronide (a major plasma metabolite). The other compounds in plasma appear to be derivatives of catechin. We therefore investigated conditions for the optimal glucuronidation of methylated and non-methylated catechin by mammalian glucuronosyltransferases and UDP-glucuronic acid. In contrast to epicatechin, we found that pooled male mouse microsomes were more efficient at glucuronidation of catechin and its derivatives than was UGT1A9, resulting in two major products from catechin initially named CG14 and CG18 based on their approximate retention times by HPLC. CG14 and CG18 were further purified by semi-preparative HPLC. Both compounds possessed a molecular ion of 465, and matched peaks in the plasma of rats fed GSPE: CG14 was a minor component, whereas CG18 was the second most abundant metabolite.

In a similar manner, glucuronidation of 3'-*O*-methyl catechin by mouse microsomes in the presence of UDP-glucuronic acid led to the formation of multiple products of which two, of retention time 19 and 24 minutes and molecular ions of 479 m/z (MCG19 and MCG24) matched metabolites in the rat plasma. Glucuronidation products of 4'-*O*-methyl-catechin did not match any of the compounds found in plasma.

2.3.4 Structural characterization of catechin and epicatechin metabolites

We have previously reported the structural characterization of 3'-*O*-methyl-epicatechin 5-*O*-glucuronide (Blount et al., 2012). In order to elucidate the structures of CG14, CG18, MCG19 and MCG24, NMR spectroscopy was performed using a Bruker Avance DRX 500 MHz spectrometer equipped with a cryoprobe. The metabolites were dissolved in CD_3OD (99.96% atom D; Sigma, St. Louis, MO) and then transferred to a 5 mm Shigemi tube (Wilmad Glass, Vineland, NJ). 2D NMR experiments using HSQC (heteronuclear correlation spectroscopy), HMBC (heteronuclear multiple bond correlation spectroscopy), and NOESY (nuclear Overhauser effect spectroscopy) were performed. The spectral data (chemical shifts) for the four compounds are shown in **Table 5**. The glucuronic acid moiety was assigned to the 7 position on (+)-catechin for CG-14 and MCG-19 based on the presence of a HMBC cross peak between the anomeric proton (H-1'') and C-7. The assignment on CG-18 and MCG-24 was to the 5 position due to a HMBC cross peak between H-1'' and C-5 (Supplemental Figure 3). The methyl group was assigned to the 3' position on MCG-19 and MCG-24 due to a NOESY cross peak between H-2' and the methoxy group, consistent with the synthesis of these compounds

from 3'-*O*-methyl catechin. **Figure 6** summarizes the structures of the four catechin metabolites, and its legend provides further description of how the positions of methylation and glucuronidation were assigned.

Table 5. NMR spectral data (chemical shifts) for CG14, CG18, MCG19 and MCG24.

CG-14	Chem. Shift (ppm)		**MCG-19**	Chem. Shift (ppm)		**MCG-24**	Chem. Shift (ppm)		**CG-18**	Chem. Shift (ppm)	
Position	^{1}H	^{13}C	Position	^{1}H	^{13}C	Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
2	4.59	82.55	2	4.63	82.54	2	4.62	83.04	2	4.59	82.53
3	3.97	68.17	3	4.01	68.29	3	4.13	66.49	3	4.05	66.31
4a	2.87	28.04	4a	2.90	30.88	4a	2.61	27.08	4a	3.00	28.35
4b	2.53	—	4b	2.54	—	4b	2.17	—	4b	2.60	—
6	6.25	96.94	6	6.22	96.94	6	6.34	97.49	6	6.27	96.76
8	6.15	96.81	8	6.14	96.59	8	6	98.08	8	6.01	97.97
2'	6.83	114.86	2'	6.97	111.46	2'	6.96	111.61	2'	6.81	114.83
5'	6.71	120.27	OMe	3.84	55.83	OMe	3.83	56.4	5'	6.70	115.75
6'	6.76	115.62	5'	6.79	115.42	5'	6.77	115.97	6'	6.75	115.50
1''	4.83	102.06	6'	6.83	121.44	6'	6.85	121.21	1''	4.84	102.23
2''	3.45	74.23	1''	4.84	102.03	1''	4.82	102.81	2''	3.48	74.43
3''	3.44	77.27	2''	3.48	74.06	2''	3.50	74.6	3''	3.48	77.43
4''	3.53	72.98	3''	3.43	74.42	3''	3.49	77.94	4''	3.55	73.12
5''	3.77	76.16	4''	3.56	72.65	4''	3.55	73.71	5''	3.81	76.57
			5''	3.87	76.13	5''	3.69	76.59			

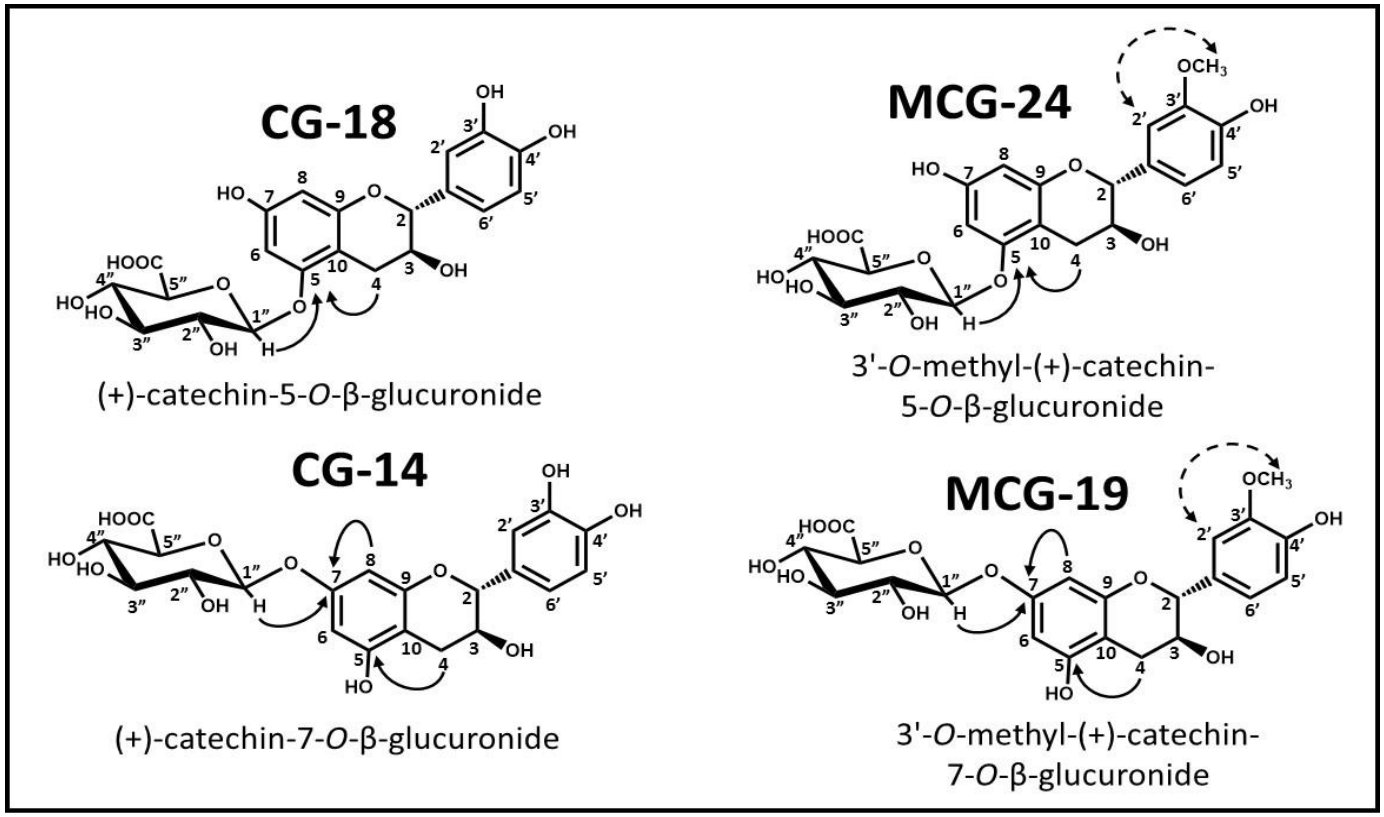

Figure 6. Structures assigned to CG14, CG18, MCG19 and MCG24. The glucuronic acid moiety was able to be assigned to the 7 position on (+)-catechin for CG-14 and MCG-19 based on the presence of a HMBC cross peak between the anomeric proton (H-1'') and C-7. The chemical shift of C-7 was determined by a HMBC cross peak between H-8 and C-7. Further support of this connectivity was provided by the absence of a cross peak between H-1'' and C-5 (chemical shift determined by a cross peak between H-4 and C-5). The assignment on CG-18 and MCG-24 was to the 5 position due to a HMBC cross peak between H-1'' and C-5. The methyl group was assigned to the 3' position on MCG-19 and MCG-24 due to a NOESY cross peak between H-2' and the methoxy group. HMBC: solid line; NOESY: dashed line

Several studies have reported the presence of PA metabolites in blood plasma after feeding animals with PA preparations or foods rich in PAs (Baba et al., 2000, 2001; Feng, 2006; Piskula and Terao, 1998; Spencer et al., 2001). The metabolites were partially identified as sulfonated, glucuronidated and/or methylated derivatives of epicatechin and catechin (Abd El Mohsen et al., 2002; Baba et al., 2000; Piskula and Terao, 1998; Spencer, 2003), with most studies focusing on the epicatechin derivatives. Definitive structures, particularly with respect to the position of glucuronidation, were not systematically assigned for many of the biologically relevant metabolites. The present systematic approach to the synthesis and identification of predominant plasma-derived metabolites of flavan-3-ols present in plasma of rodents dosed with GSPE provides a blueprint for the development of improved methods for the evaluation of bioavailability of bioactive polyphenols, as well as a source of standard target molecules for evaluating modes of action.

2.3.5 Cross-validated analytical method for determining plasma levels of catechin and epicatechin metabolites

LC-MS/MS using an Agilent 6460 Triple Quadrupole system was selected as the analytical method to determine the levels of catechin and epicatechin metabolites in samples of plasma from rats fed GSPE, due in part to the sensitive and selective nature of this method as well as the broad application of this method in the literature (Roura et al., 2008; Tsang et al., 2005; Urpi-Sarda et al., 2009). Initially, the linear dynamic range for each molecule (catechin, epicatechin, two catechin glucuronides, two methyl-

catechin glucuronides and one methyl-epicatechin glucuronide) was determined for concentrations ranging over 4 orders of magnitude (pM-µM), with lowest concentrations in the 250-650 pM range (see **Figure 7** for representative chromatograms). The linear dynamic range was observed to be excellent (**Table 6**) and provides the ability to quantify these compounds across a range of concentration typical in biological fluids and tissues. Metabolites were extracted from plasma by solid phase extraction (extraction recovery was estimated to be 96.0 ± 3.1% based on internal standard recovery) as described in Materials and Methods.

Measurement of effects of matrix and plasma concentration, and intraday and interday variability revealed the method to be highly robust and efficient at limiting ion suppression or other matrix effects commonly reported in analysis of biological samples(Gardana et al., 2007; Li et al., 2007; Matuszewski et al., 2003), but rarely accounted for. As expected, there was a greater matrix effect at lower concentrations. The % ME when measuring catechin ranged from -7.84% at 500nM to -20.7% at 50nM. MCG24 ranged from -13.5% at 500nM to -23.0 at 50nM. Finally, CG18 ranged from -13.1% at 500nM to -15.7% at 50nM. Since metabolites appear in rodent plasma at approximately 300-400nM after repeated dosing of GSPE, using the SPE procedure results in underestimating metabolites by about 13%.

Table 6. Linear dynamic ranges for the quantitation of (methyl)catechin and (methyl)epicatechin glucuronides by LC-MS/MS.

Compound	Range	Regression line	Linearity (R^2)	LOQ (S/N=5) MOC	LOD (S/N=3) MOC
C	5nM-50µM	y = 7E+09x+ 528.31	0.9997	138.9 pmol	55.5 pmol
EC	5nM-50µM	y = 7E+09x+ 689.79	0.9997	106.4 pmol	48.4 pmol
3'OMe-EC-5-O-glucr	250pM-2.5µM	y=5E+11x– 3699.8	0.9998	2.05 fmol	1.07 fmol
CG18	500pM-10µM	y = 4E+11x+ 403.99	0.9999	10.6 fmol	3.41 fmol
CG14	500pM-10µM	y=6E+11x– 16601	0.9998	3.68 fmol	1.2 fmol
MCG19	650pM-10µM	y=2E+11x+ 12580	0.9990	5.80 fmol	2.80 fmol
MCG24	500pM-20µM	y=4E+11x– 31920	0.9994	4.70 fmol	1.03 fmol

The Horowitz ratio (HorRat), a well-recognized normalized performance parameter that provides a measure of laboratory precision, was calculated for individualized metabolites based on the equation described by Horwitz and Albert (Horwitz and Albert, 2006). HorRat values ranging from 0.13 to 1.29 indicated the high reproducibility of this method. While lower values of the HorRat (0.13-0.21) do fall outside of the generally accepted range, it is important to note that these were for minor metabolites (C-7-*O*-glucuronide, 3'-*O*-Me-C-7-*O*-glucuronide). Comparatively, values for major metabolites identified in this method, (C-5-*O*-glucoronide and 3'-*O*-Me-5-*O*-glucornide) were 1.29 and 0.51, respectively. Overall these data are consistent with that of a highly reproducible analytical method.

Calculated LOD (defined as 3:1 signal:noise) ranged from 1.03-3.41 fmoles on column for metabolites of catechin and epicatechin compared to 55.5 and 48.4 pmoles on column for parent compounds catechin and epicatechin, respectively. This suggests the potential for more efficient ionization of metabolites relative to parent compounds. These values translated to LOQs of 2.1-10.6 fmol on column or ~11-53 fmol per mL of plasma for metabolites.

Finally, the analyses were repeated at a second site, using the identical column and separation parameters, but a different mass spectrometer (see Materials and Methods). The C and EC metabolites in the plasma were resolved by HPLC with very similar profiles to those recorded previously. Calibration with authentic standards resulted in absolute values and standard deviations that closely matched the results from the first site analyses, with the exception of MCG24 (3'-*O*-Me-C-5-*O*-glucoroide),

which was determined based on calibration with the corresponding 7-*O*-gluconide. It was observed that the C and EC metabolites were unstable in thawed plasma samples, as recently reported elsewhere (Zhang et al., 2013), such that reproducible results were only obtained if the samples were injected onto the HPLC within a few hours. Samples left at room temperature were almost totally degraded after 3 days.

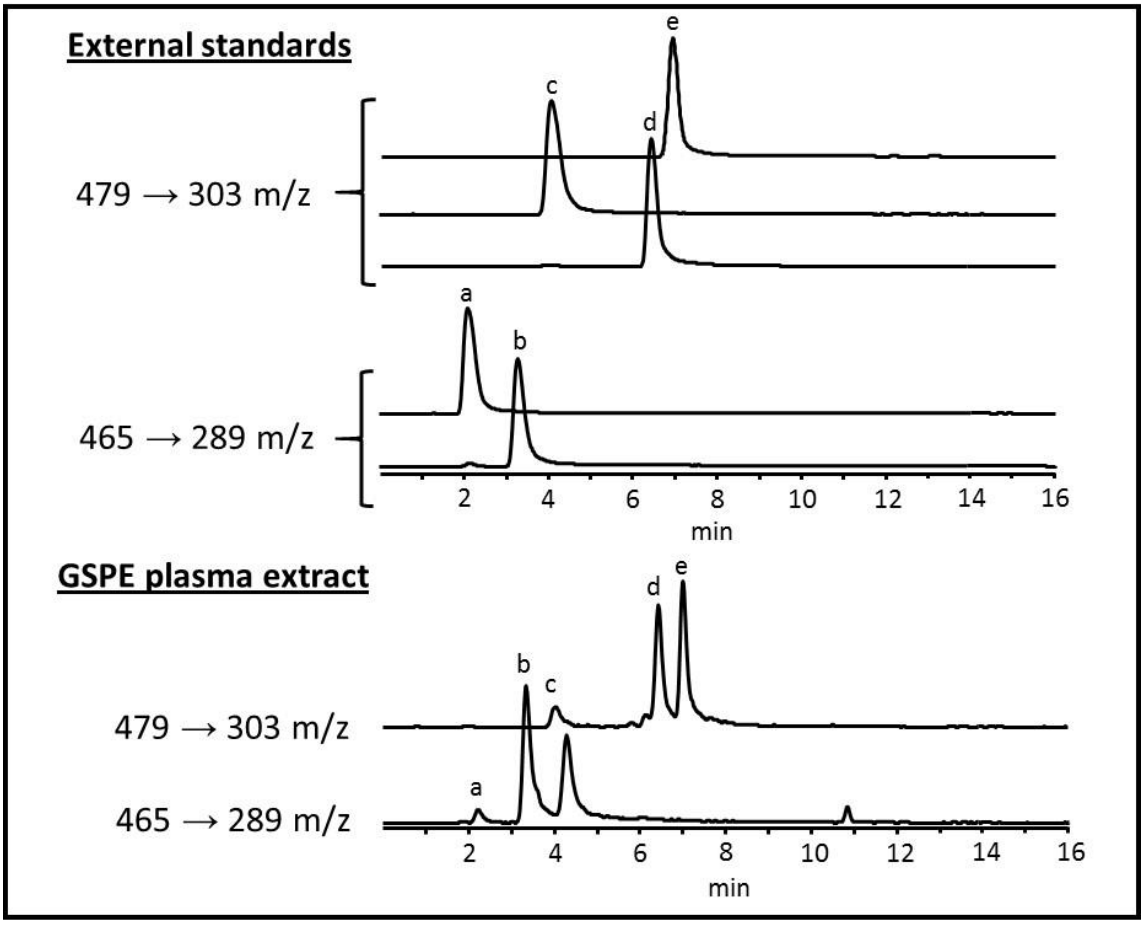

Figure 7. Chromatographic separation of and identification of C/EC metabolites for each mass transition. Peak identification: (a) CG14, 7-glucr; (b) CG18, 5-glucr; (c) MCG19, 7-glucr; (d) MCG24, 5-glucr; (e) 3'OMe-EC-5-glucr.

2.3.6 Advantages and Disadvantages of the Present Approach

Alternative approaches for identifying metabolites of flavan-3-ols in biological fluids have wither generated standard compounds by chemical synthesis (González-Manzano et al., 2009) or attempted to identify the compounds directly from the fluids (Abd El Mohsen et al., 2002; Natsume et al., 2003; Piskula and Terao, 1998; Spencer, 2003); most of these previous approaches have only unequivocally identified a subset of the metabolites present. A recent study has reported the chemical synthesis and full characterization of a complete range of EC glucuronides and sulfates (Zhang et al., 2013). Such compounds will be of great value for future studies on bioavailability, although their chemical synthesis is complex, requiring as a starting point the availability of a complete series of orthogonally protected EC derivatives.

The present study as combined a semisynthetic/biosynthetic scheme with matching of compounds to authentic metabolites. Although the glucuronosyl transferase enzymes are not completely regionspecific their mammalian origin means that they can generate physiologically relevant metabolites, and the matching of synthetic compounds to plasma metabolites prior to final purification means that only those metabolites that are physiologically relevant are pursued. Furthermore, an enzymatic strategy has advantages of the facile introduction of radiolabel, for examples, in the methyl (from S-adenosyl-L-methionine) or glucuronide (from UDP-glucuronic acid) moieties, should labeled compounds be required for mechanism of action studies or analytical internal standards. The limiting step from a cost perspective are the price of the enzymes, which cannot by recycles, and the cost of UDP-glucuronic acid. Now that

standards of all glucuronidated metabolites are available for subsequent identification of products, this problem could be circumvented by a total biological synthesis using recombinant enzymes express in *E. coli*, as we have previously shown for synthesis of flavonol and isoflavone glucosides (He et al., 2008). It should be notes that the present work does not address the sulfated derivatives that also arise from mammalian metabolites of flavan-3-ols , particularly in humans (Ottaviani et al., 2012).

CHAPTER 3. DIFFERENTIATED CACO-2 CELL MONOLAYERS EXHIBIT ADAPTATION IN TRANSPORT AND METABOLISM OF FLAVAN-3-OLS WITH CHRONIC EXPOSURE TO BOTH ISOLATED FLAVAN-3-OLS AND ENRICHED EXTRACTS

As part of the manuscript submitted to Food & Function, BW Redan and MG Ferruzzi,

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3.1 Introduction

Flavan-3-ols are key flavonoids widely distributed in the food supply and are naturally abundant in foods such as tea, cocoa, and grape. These compounds include monomeric forms such as catechin (C) and epicatechin (EC), along with complex proanthocyanidin (PAC) oligomers and polymers, present in grape and cocoa. Additionally, gallated forms including epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) are abundant in tea. Data from epidemiological studies supports the view that diets rich in flavan-3-ols are associated with a decreased risk of specific chronic diseases including cardiovascular disease (Tresserra-Rimbau et al., 2014), diabetes (Zamora-Ros et al., 2013a), and certain types of cancers (Zamora-Ros et al., 2013b). Supporting these epidemiological associations, flavan-3-ols, and especially EC, have demonstrated ability to improve clinical markers related to cardiovascular function such

as blood pressure, cholesterol, and flow-mediated dilation (Hooper et al., 2012; Sansone et al., 2015). However, translation of flavan-3-ols for use in disease-preventative strategies is limited by several factors including the notion that, excluding microbial metabolites, <5% of the parent compound is typically absorbed from an oral dose (Neilson and Ferruzzi, 2011). This is particularly true in the case of bioavailability assessed from an acute dose, the most predominate experimental paradigm.

While acute bioavailability studies are most common, evidence does exist that longer-term exposure to flavan-3-ols that is more representative of widespread dietary exposure or chronic consumption of flavan-3-ol-rich foods can significantly enhance overall bioavailability in humans and experimental animals. Ten day administration of a grape seed extract product (GSE) to Sprague-Dawley rats was found to improve plasma response of C and EC by greater than 250% (Ferruzzi et al., 2009). Similarly, chronic GSE treatment in rodents increased phase II metabolites as much as 600-700% for C/EC glucuronides and approximately 140-240% for methyl-C/EC-glucuronides (Wang et al., 2012). In addition, clinical research has demonstrated that 4 week chronic administration of 800 mg EGCG per day before a pharmacokinetic assessment results in EGCG plasma AUC values 60% greater than those not given EGCG (Chow et al., 2003). Collectively these data suggest that single acute pharmacokinetic assessments may not fully represent the complex response observed from longer-term dietary exposure. Having been observed in both pre-clinical and clinical paradigms, the implication of this adaption remains to be better understood and perhaps leveraged in a manner

consistent with understanding the bioavailability of flavan-3-ols from longer-term dietary patterns.

While the exact mechanisms behind this phenomenon are not completely understood, what is known is that upper intestinal absorption and metabolism of flavonoids rely on both inducible and non-inducible metabolizing and transport systems (Croom, 2012) that may be impacted by previous exposure to these compounds. Since the gastrointestinal tract is the main barrier to delivery of flavonoids, we hypothesize that adaptation occurring in the upper gastrointestinal epithelium is largely controlling absorption of ingested compounds and may be partially responsible for the observed increase in flavan-3-ol absorption and metabolism with chronic exposure. Though gut microbiota have been reported to affect systemic delivery of phenolic compounds (Tomás-Barberán et al., 2014), we are focusing here on parent flavonoids and their Phase II metabolites since their relevance to bioactivity has been more widely studied.

Differentiated Caco-2 monolayers are highly predictive of human absorption and have been employed to study absorption of a number of pharmaceuticals and phytochemicals because they form enterocyte-like cells (Sambuy et al., 2005). However, many studies involving the Caco-2 model are limited to acute studies (Hubatsch et al., 2007) and do not consider how chronic exposure typical of dietary patterns modulates cellular processes associated with transport across the gut epithelium. Therefore, Caco-2 cell monolayers were exposed throughout differentiation to isolated flavan-3-ols and extracts rich in flavan-3-ols to determine if adaptation in transport and metabolism of

these compounds could be induced in this widely-utilized model to better mimic the *in vivo* phenomenon.

3.2 Materials and Methods

3.2.1 Materials

Authentic standards of (-)-epicatechin, (+)-catechin, (-)-epigallocatechin gallate, and (-)-epigallocatchin were obtained from Sigma (St. Louis, MO). Teavigo® (>90% EGCG) was used for cell culture treatments. Green tea extract was provided by Nestlé (Marysville, OH) and grape seed extract (MegaNatural-BP®, Polyphenolics, Madera, CA) was purchased from an online retailer. Solvents for liquid chromatography were of mass spectrometry grade and all other reagents were either cell culture or at least analytical grade.

3.2.2 Cell culture and treatments

Caco-2 TC7 cell line (passages 81-84) was maintained in DMEM media with 10% v/v FBS, 1% v/v nonessential amino acids, 1% v/v HEPES, 1% v/v penicillin/streptomycin, and 0.1% v/v gentamicin. Cells were incubated at 37°C under a 5% CO_2/95% air atmosphere at constant humidity. For two compartment model experiments, cells were seeded at a density of 3.2 x 10^4 cells/mL onto 6-well plates and allowed to differentiate over a period up to 12 d post-confluency. For cells cultured on Transwell® inserts (Corning® polyester membrane, 24 mm diameter, pore size 0.4 µm), cells were seeded at a density of 2.12 x 10^5 cells/cm^2 and differentiated over a period of 21-25 d post-confluency. Cells treated with isolated compounds were cultured in growth media with

0 µM (control), 1 µM, or 10 µM of EC or EGCG added to the apical chamber (see **Figure 8** for outline of experimental design). Preliminary experiments indicated that cell monolayers could be treated with these concentrations without significantly decreasing cell viability (>95%) as determined by MTT assay (Biotium, Hayward, CA). Cells treated with extracts were cultured in growth media containing GTE rich in EGCG and ECG or GSE rich in C and EC monomers and polymers. Media was prepared using the same concentrations as pure compounds as determined by the Folin-Ciocalteu total phenolic method (Waterhouse, 2001) and changed every 48 h.

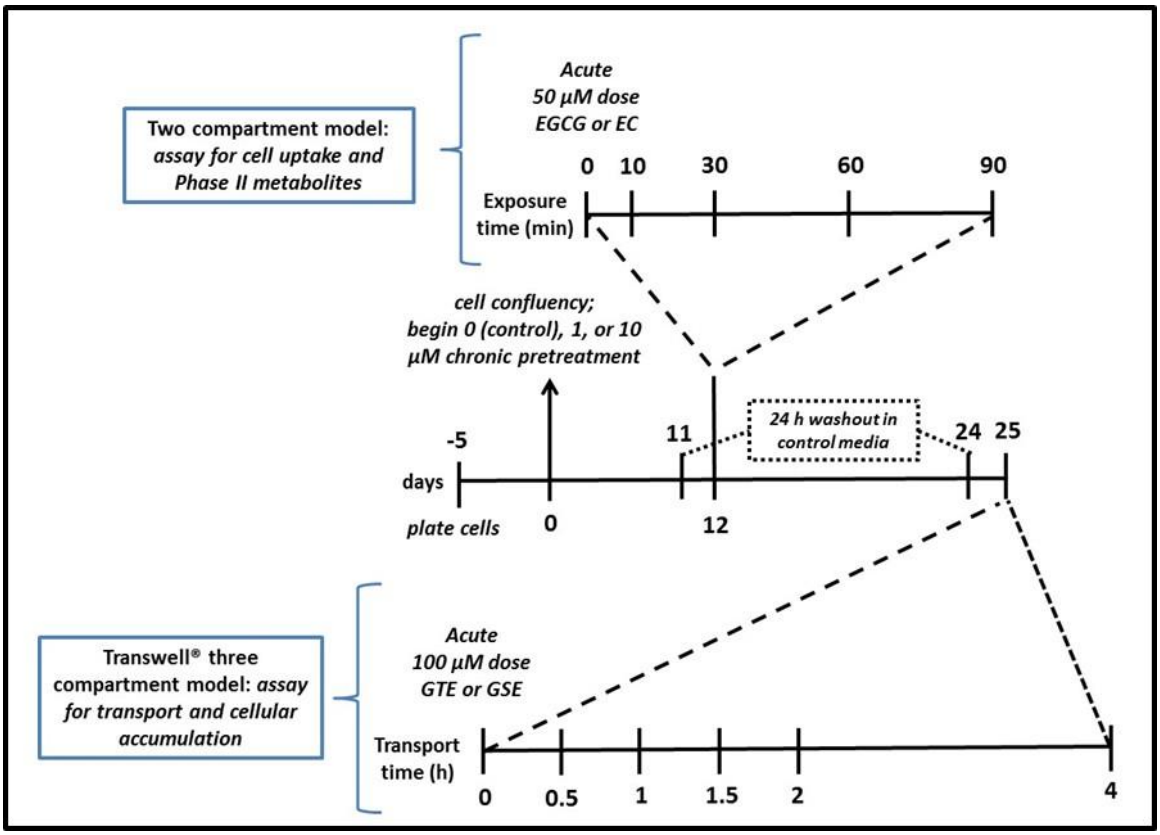

Figure 8. Experimental design to simulate chronic treatment of Caco-2 cell monolayers. Cells were cultured in growth media with either 0 µM (control), 1 µM, or 10 µM of EGCG, EC, green tea, or grape seed extract added to the apical chamber. After all cell treatments were cultured in control media for 24 h, transport was assessed over 4 h in the three compartment model with a 100 µM acute dose of green tea or grape seed extract, while uptake and metabolism was assessed over 90 min in the two compartment model using a 50 µM acute dose. Abbreviations: GTE, green tea extract; GSE, grape seed extract; EGCG, epigallocatechin gallate; EC, epicatechin.

3.2.3 Flavan-3-ol transport, uptake, and metabolism

After a 24 h washout period in control growth media, cell monolayers were rinsed with 0.1% fatty-acid free albumin in PBS, followed twice with PBS only. Uptake and metabolism of pure compounds was assessed by an acute dose of 50 µM EC or EGCG in PBS (adjusted to pH = 5.5), and cells were harvested at 0 (media immediately removed after loading onto monolayer), 10, 30, 60, and 90 min. Transport of GTE and

GSE flavan-3-ols using the Transwell® system was assessed by an acute dose of 100 µM total phenolics in PBS (pH = 5.5). 1 mL basolateral media was collected and replaced with fresh PBS at 0, 15, 30, 60, 120, and 240 min. Experiments were performed in triplicate. After the uptake period, cells were rinsed according to the above procedure and then collected in ice cold PBS (pH = 5.5). The bicinchoninic acid (BCA) method using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to determine protein levels.

3.2.4 Cell extraction and transport media preparation

Cells were lysed by sonification for 10 s, after which 1 mL of PBS and MeOH were added. Cells were extracted three times with 0.01% w/v BHT/ethyl acetate, organic layers were pooled, and then evaporated under a nitrogen stream. The extract was resolubilized in 100 µL of 80:20 0.4% formic acid (aq.): acetonitrile (0.1% formic acid) (v:v) and then centrifuged at 18,000 *g* for 5 min to remove particulates before injecting 20 µL in the LC-MS-TOF system. Transport media was acidified with 1% v/v formic acid/acetonitrile, left on ice for 10 min to precipitate proteins, centrifuged, and injected as described above.

3.2.5 Characterization of flavan-3-ols and metabolites

Parent compounds and metabolites were separated using a Waters RP-C18 column (Xbridge BEH Shield, 2.5 µm, 2.1x100 mm) and characterized using an Agilent 1100 high-pressure liquid chromatography system in tandem with a Waters LCT Premier

time-of-flight mass spectrometer (LC-TOF-MS) with electrospray ionization (ESI) in negative mode. Flow was set to 0.25 mL/min and mobile phases were A: 0.4% formic acid (aq.); B: acetonitrile (0.1% formic acid). Gradient conditions were 5% B at 0 min, 35% B at 15 min, 70% B at 17 min, 5% B at 19 min, and 5% B at 23 min to reset the gradient. Extracted ion chromatograms (EICs) *m/z* are shown in **Tables 7** and **8**. Standard curves were produced using standards of parent compounds (C, EC, EGCG, and EGC) and were used to quantify intracellular/transported compounds in addition to estimating intracellular metabolite concentrations.

Table 7. Green tea (GTE) and grape seed extract (GSE) transport loading media flavan-3-ol composition (100 µM total phenolics). Values shown are displayed as average ± SD (n = 3).

Compound	*m/z* [M-H]$^-$	GTE µM ± SD	GSE µM ± SD
Catechin (C)	289	4.83 ± 0.48	3.25 ± 0.62
Epicatechin (EC)	289	21.9 ± 1.0	6.34 ± 1.23
Epigallocatechin (EGC)	305	35.2 ± 1.9	Not quantified
Epicatechin gallate (ECG)	441	0.94 ± 0.050	Not detected
Epigallocatechin gallate (EGCG)	457	24.1 ± 1.2	Not detected
Proanthocyanidin dimer B2 (PAC B2)	577	Not detected	2.49 ± 0.42
TOTAL		86.9	12.1

3.2.6 Data analysis

Values for cellular accumulation of flavan-3-ols and metabolites are expressed as average pmol ± SEM. Linear regression using Excel was performed to determine the slope for use in rate calculations (Microsoft, Redmond, WA). The apparent permeability coefficient (P_{app}) was calculated using an equation for use in Caco-2 transport studies (Hubatsch et al., 2007). Analysis was performed using SAS 9.3 (SAS Institute, Cary, NC),

and significant differences were evaluated by a Bonferroni post-hoc pairwise test (α = 0.05).

3.3 Results

3.3.1 Monomeric flavan-3-ol composition of extracts and stability to experimental conditions

GTE shows a similar profile of flavan-3-ols as previously reported (Neilson et al., 2010), with the major compounds being EGCG and EGC (Table 7). Concentrations of these compounds in this extract though appear to have lower amounts of gallated with respect to non-gallated flavan-3-ols. Average recovery of flavan-3-ols over 4 h in cell-free transport media was 92.1%. Table 7 shows the concentrations of C, EC, and PAC B2 in the transport media, which was within the range of that previously reported (Villani et al., 2015). Average recovery of these flavan-3-ols over 4 h in cell-free transport media was 79.5%.

3.3.2 Differential cellular uptake and metabolism of flavan-3-ols

In addition to unmetabolized EGCG and EC, methylated and sulfonated metabolites of these compounds were detected in Caco-2 cells. **Figure 9** shows representative chromatograms of intracellular EGCG, EC, and their metabolites, which is similar to previous reports of Caco-2 cell metabolism of flavan-3-ols (Sanchez-Bridge et al., 2015; Zhang et al., 2004). After 30 min, 1 µM EGCG pretreated monolayers showed significantly ($P < 0.05$) greater accumulation of EGCG compared to control (80.8 vs. 189

pmol/mg protein; control vs. 1 μM pretreatment), but no other differences in accumulation were observed (**Figure 10**). While there was no alteration in the rate of EGCG uptake or methylated EGCG appearance with treatments, 10 μM EGCG pretreatment decreased appearance rate of both sulfated (0.27 vs. 0.063 pmol·min^{-1}·mg protein^{-1}; control vs. 10 μM pretreatment; $P < 0.01$) and methyl sulfate derivatives (0.23 vs. 0.070 pmol·min^{-1}·mg protein^{-1}; control vs. 10 μM pretreatment; $P < 0.05$) compared to control (**Table 8**).

After 60 min, both 1 and 10 μM EC pretreatments resulted in significantly ($P < 0.05$) greater EC accumulation compared to control (221 pmol/mg protein, control; 376 pmol/mg protein, 1 μM pretreatment; 352 pmol/mg protein, 10 μM pretreatment), with the 1 μM pretreatment remaining significantly greater after 90 min (273 vs. 569 pmol/mg protein; control vs. 1 μM treatment). Similarly, both treatments displayed significantly ($P < 0.05$) greater levels of methylated EC derivatives after 60 min (38.5 pmol/mg protein, control; 64.4 pmol/mg protein, 1 μM pretreatment; 71.2, 10 μM pretreatment; pmol/mg protein), with the 10 μM pretreatment showing approximately twice the concentration of methylated EC at 90 min (46.5 vs. 101 pmol/mg protein; control vs. 10 μM pretreatment; $P < 0.05$). Rate of cell accumulation of EC and its metabolites over 90 min showed that while 10 μM pretreated monolayers had a significantly increased rate of EC methylation (0.43 vs. 1.03 pmol·min^{-1}·mg protein^{-1}; control vs. 10 μM pretreatment; $P < 0.01$) there was decreased appearance of sulfated derivatives (0.90 vs. 0.53 pmol·min^{-1}·mg protein^{-1}; control vs. 10 μM pretreatment; $P <$

0.05). No significant differences were observed in the rate of appearance of methylated-EC sulfate metabolites with pretreatment.

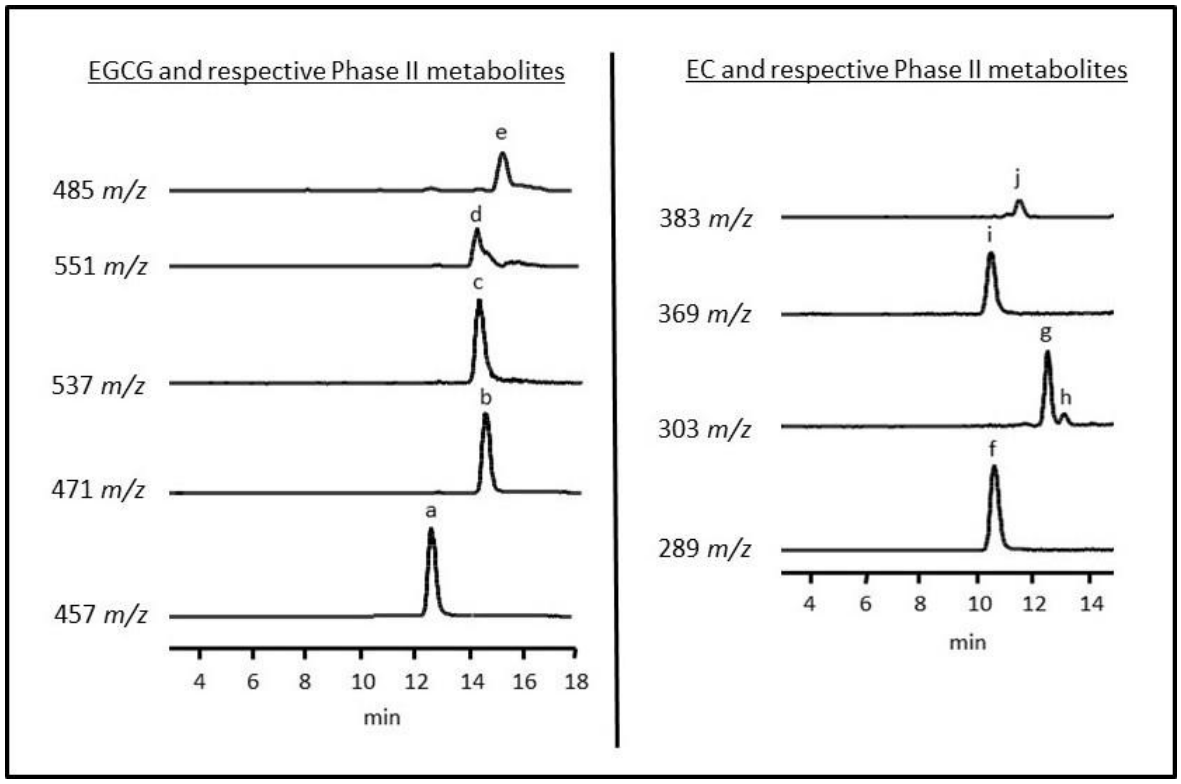

Figure 9. Representative chromatograms of intracellular EGCG, EC, and their respective metabolites. Tentative identification of peaks: a, EGCG; b, Me-EGCG; c, EGCG-S; d, Me-EGCG-S; e, dimethyl-EGCG; f, EC; g, 3'methyl-*O*-EC; h, 4'-*O*-methyl-EC; i, EC-S; j, Me-EC-S.

3.3.3 Differential alteration in transport of flavan-3-ol-rich extracts across Caco-2 monolayers

Since major flavan-3-ol Phase II metabolites of the parent compounds were not observed to a great extent in the three compartment model, we focused on tracking the parent compounds for flux through the cell monolayers. **Figure 11** shows that after 4 h, 10 µM GTE pretreatment resulted in 38% greater cumulative transport of EC compared

to control (12.1 vs. 16.7 nmol; control vs. 10 µM pretreatment; $P < 0.05$). GTE pretreatment resulted in significant ($P < 0.05$) differences in transport of gallated flavan-3-ols at 60 min, with approximately 60% greater cumulative transport of EGCG (0.56 vs. 0.89 nmol; control vs. 1 µM pretreatment) and a 44-61% increase in ECG transport (0.16 nmol, control; 0.26 nmol, 1 uM pretreatment, 0.23 nmol, 10 uM pretreatment). For monolayers pretreated with 10 µM grape seed extract, there was significantly ($P < 0.05$) greater cumulative transport of C (2.0 vs. 3.1 nmol; control vs. 10 uM pretreatment), EC (5.4 vs. 6.3 nmol; control vs. 10 uM pretreatment), and PAC B2 (1.0 vs. 1.5 nmol; control vs. 10 uM pretreatment) after 4 h (see Figure 11). No significant differences were observed in cellular accumulation of flavan-3-ols with tea or grape seed extract treatments (data not shown).

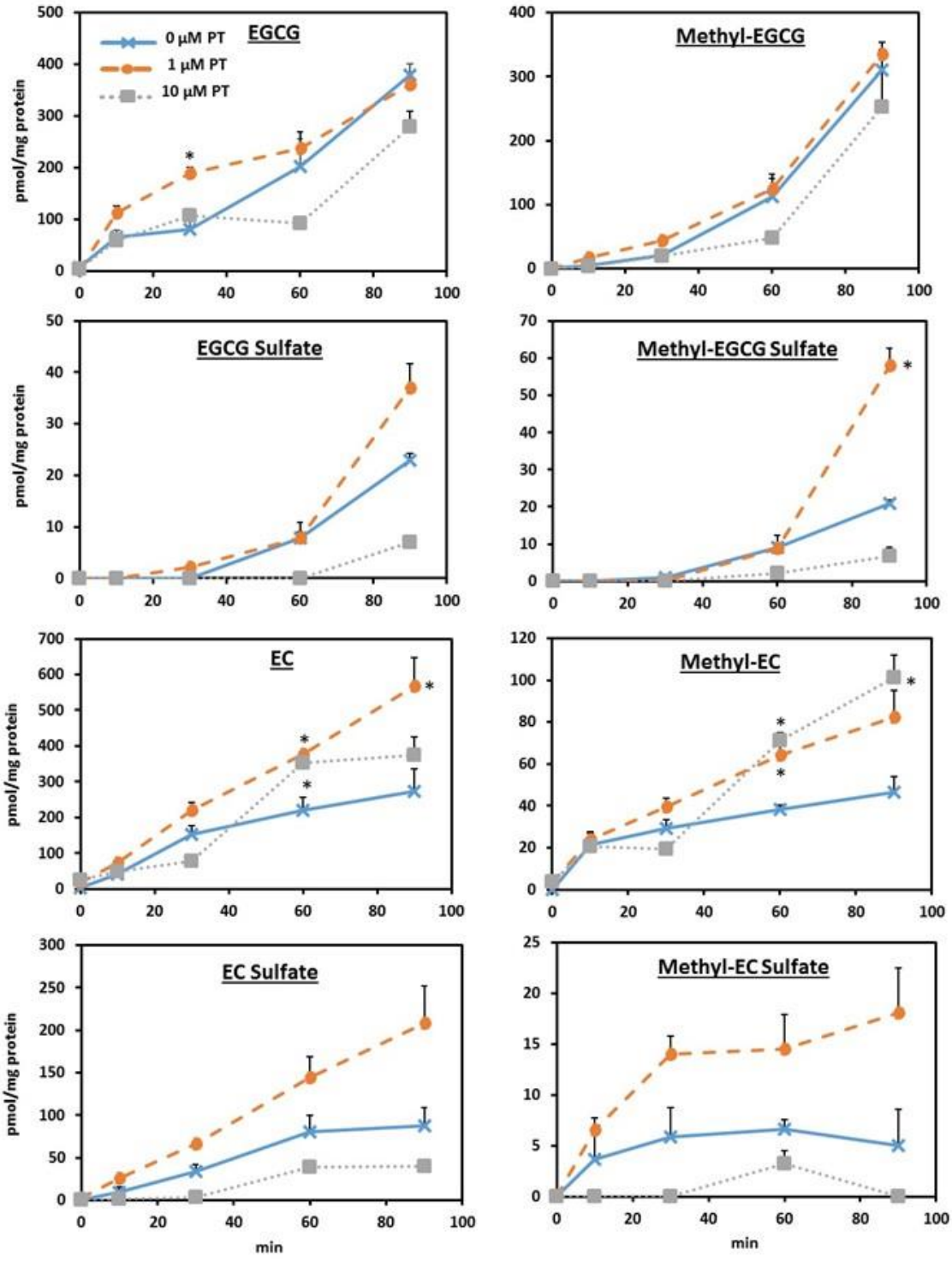

Figure 10. Effect of pretreatment on Caco-2 uptake and metabolism of EGCG and EC over 90 min was assessed using monolayers differentiated in a 6-well two compartment model. Monolayers were exposed to either 0 µM (control), 1 µM or 10 µM EGCG or EC pretreatment (PT) in growth media following the paradigm and conditions described in

(Figure 10 caption, con'd.) Parent compounds, along with the methylated and sulfonated metabolites were identified by LC-TOF-MS. *$P < 0.05$ compared to control.

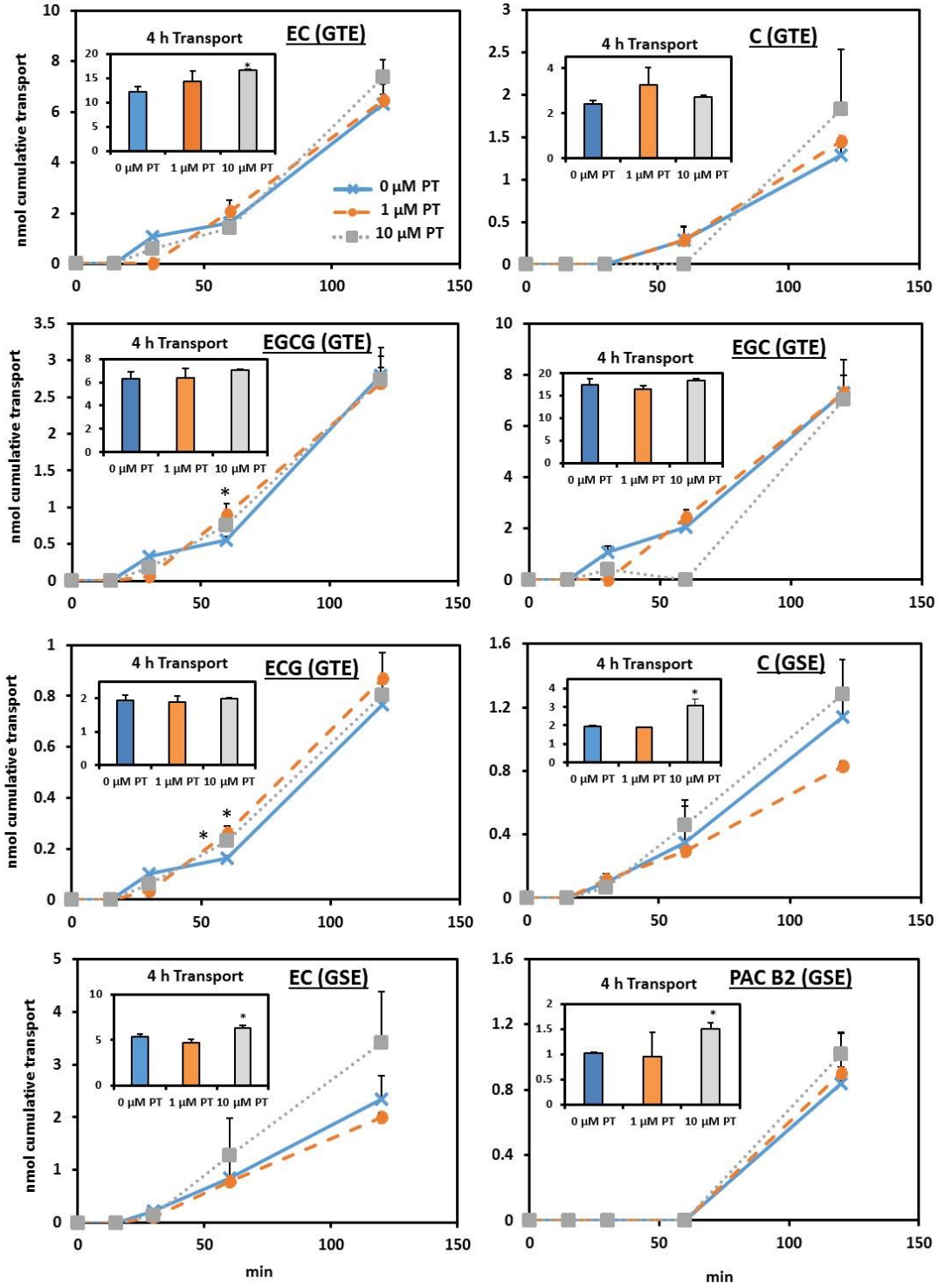

Figure 11. Chronic pretreatment with green tea extract differentially alters

(Figure 11 caption, cont'd.) apical to basolateral transport of flavan-3-ols across differentiated Caco-2 cell monolayers. Confluent Caco-2 monolayers were cultured on Transwell® inserts in pretreatment (PT) media containing either 0 µM (control), 1 µM, or 10 µM total green tea or grape seed phenolics (see Table 1 for composition of transport media) during the differentiation process. All cell monolayers were incubated with control media 24 h before transport was assessed using a 100 µM total phenolics acute dose of total green tea or grape seed extract over 4 h. Statistical pairwise comparison was performed comparing 0 µM (control) to treatments. *P < 0.05 compared to control. Abbreviations: PT, pretreatment; C, catechin; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; PAC B2, proanthocyanidin B2.

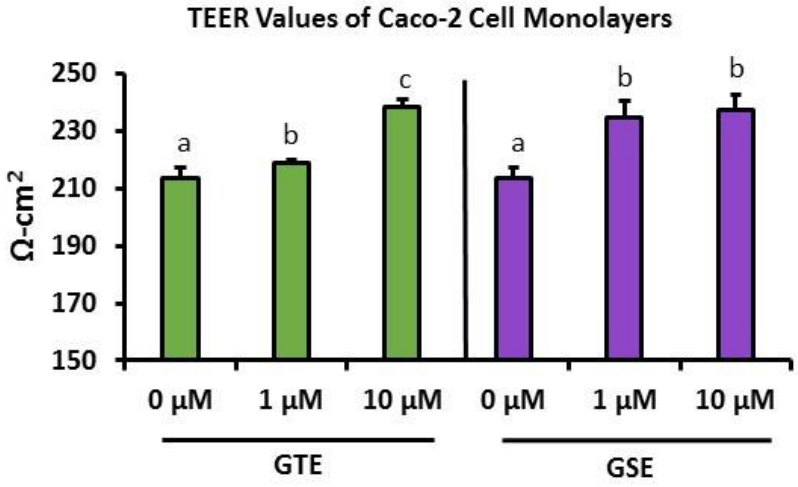

Figure 12. Pretreatment Increases Transepithelial Electrical Resistance (TEER) values**.** Caco-2 cells grown on Transwell® inserts (n = 3) were pretreated with either 0 (control), 1, or 10 µM total phenolics of green tea extract (GTE) or grape seed extract (GSE) indicating an increase in formation of cellular tight junctions. Differing letters indicate significant difference (*P* < 0.05) within treatment type.

Table 9 displays the apparent permeability coefficient (P_{app}) calculated from the transcellular flux of each parent flavan-3-ol compound from green tea and grape seed extracts. After 2 h, 1 µM GTE pretreated monolayers showed greater ($P < 0.01$) P_{app} of C (7.05×10^7 cm/s vs. 4.93×10^7 cm/s; control vs 1 µM pretreatment) and EC (5.23×10^7 cm/s vs. 7.64×10^7 cm/s; control vs 1 µM pretreatment) compared to control, though there was a tread in significance for increased EC P_{app} with 10 µM pretreatment ($P = 0.0599$). Similarly, significant ($P < 0.05$) differences in C transport were observed over 2 h in monolayers receiving 10 µM GSE pretreatment (6.54×10^7 cm/s vs. 7.46×10^7 cm/s; control vs 1 µM pretreatment). Additionally, there was a trend ($P = 0.0515$) for increased PAC B2 P_{app} over 4 h with 10 µM GSE pretreatment. Interestingly, 10 µM pretreatment with both green tea and grape seed extracts significantly ($P < 0.05$) increased transepithelial electrical resistance (TEER) of differentiated monolayers compared to control (**Figure 12**).

Table 8. Rate (pmol/min) of differentiated Caco-2 cell accumulation of EGCG/EC and formation of EGCG/EC Phase II metabolites in pretreated monolayers compared to control.[a]

Compound	*m/z* [M-H]$^-$	Pretreatment	Rate of cell accumulation ± SEM (pmol·min^{-1}·mg protein^{-1}) over 90 min
EGCG	457	0 µM	3.67 ± 0.334
		1 µM	3.67 ± 0.337
		10 µM	2.67 ± 0.334
Methyl-EGCG	471	0 µM	3.33 ± 0.334
		1 µM	3.67 ± 0.334
		10 µM	3.00 ± 0.578
EGCG Sulfate	537	0 µM	0.267 ± 0.0334
		1 µM	0.367 ± 0.0667
		10 µM	0.0633 ± 0.0120**
Methyl-EGCG Sulfate	551	0 µM	0.233 ± 0.0334
		1 µM	0.600 ± 0.0578**
		10 µM	0.0700 ± 0.0173*
EC	289	0 µM	3.00 ± 0.578
		1 µM	6.00 ± 1.01$^{\#}$
		10 µM	5.00 ± 4.33
Methyl-EC	303	0 µM	0.433 ± 0.0667
		1 µM	0.833 ± 0.120*
		10 µM	1.03 ± 0.0334**
EC Sulfate	369	0 µM	0.900 ± 0.0578
		1 µM	2.00 ± 0.578
		10 µM	0.533 ± 0.0667*
Methyl-EC Sulfate	383	0 µM	0.0533 ± 0.0241
		1 µM	0.153 ± 0.0467
		10 µM	0.0120 ± 0.00405

[a]Values are presented as average (n = 3) ± SEM. Statistical pairwise comparison is between pretreatments and 0 µM. *P<0.05; **P<0.01; $^{\#}P$=0.0602.

Table 9. Apparent permeability coefficients (P_{app}) of green tea (GTE) or grape seed (GSE) extract flavan-3-ols across differentiated Caco-2 cell monolayers.[a]

Compound	Pretreatment	P_{app} ± SEM (x 10^7 cm/s) over 2 h	P_{app} ± SEM (x 10^7 cm/s) over 4 h
C (GTE)	0 µM	4.93 ± 0.336	6.32 ± 0.274
	1 µM	7.05 ± 0.0966**	6.48 ± 1.30
	10 µM	8.45 ± 3.24	5.86 ± 0.382
EC (GTE)	0 µM	5.23 ± 0.389	6.57 ± 0.652
	1 µM	7.15 ± 0.117**	6.28 ± 0.798
	10 µM	7.64 ± 0.834$^{\#}$	7.18 ± 0.105
EGCG (GTE)	0 µM	2.64 ± 0.322	2.47 ± 0.216
	1 µM	2.68 ± 0.370	2.51 ± 0.314
	10 µM	2.65 ± 0.157	2.72 ± 0.0715
ECG (GTE)	0 µM	9.18 ± 0.896	9.44 ± 0.801
	1 µM	10.8 ± 1.28	9.37 ± 1.03
	10 µM	9.87 ± 0.666	9.76 ± 0.211
EGC (GTE)	0 µM	4.80 ± 0.809	4.61 ± 0.243
	1 µM	5.04 ± 0.438	4.48 ± 0.117
	10 µM	4.97 ± 0.348	5.24 ± 0.163$^{\#\#}$
C (GSE)	0 µM	6.54 ± 0.441	5.79 ± 0.285
	1 µM	4.75 ± 0.0912	5.47 ± 0.0237
	10 µM	7.46 ± 1.39*	8.79 ± 1.04
EC (GSE)	0 µM	6.94 ± 0.980	7.90 ± 0.101
	1 µM	6.01 ± 0.222	6.89 ± 0.549
	10 µM	10.3 ± 3.05	9.47 ± 0.651
PAC B2 (GSE)	0 µM	6.02 ± 0.703	4.23 ± 0.0297
	1 µM	6.45 ± 1.79	3.84 ± 1.96
	10 µM	7.31 ± 0.896	6.07 ± 0.453†

[a]Values are presented as average (n = 3) ± SEM. Statistical pairwise comparison is between pretreatments and 0 µM. *P<0.05; **P<0.01; $^{\#}P$=0.0588; $^{\#\#}P$=0.0991; $^{\dagger}P$=0.0515.

3.4 Discussion

The Caco-2 human intestinal cell model is commonly used to investigate factors impacting absorption and to a certain extent metabolism of dietary flavonoids. However, typical experimental paradigms used in cell-based studies rely on acute exposures that may not mimic chronic dietary exposure to flavonoids. The extent to which the Caco-2 model can exhibit adaptive responses similar to those observed in humans and experimental animals is critical to establish in order to enhance the predictive nature of this important model. Our data suggest Caco-2 cell human intestinal cell monolayers may, in fact, adapt differentially to transport of gallated versus non-gallated flavan-3-ols with exposure to these phenolics during differentiation. While non-gallated flavan-3-ols (C and EC) generally exhibited greater levels of cumulative transport at later time points with pretreatment (2-4 h; see Figure 10), gallated flavan-3-ols (EGCG and ECG) showed differences in transport and accumulation at 30 and 60 min time points (See Figures 10 and 11). Since gallated flavan-3-ols are less bioavailable as compared to non-gallated,(Zhang et al., 2004) these points where alterations in transport and accumulation occurred may be a reflection of different absorption efficiencies or perhaps transport mechanisms being affected by chronic exposure to gallated and non-gallated flavan-3-ols. Similarly, Caco-2 cell monolayers pretreated with grape anthocyanins for 96 h exhibit approximately 50% greater transport of these compounds compared to untreated cells (Faria et al., 2009), indicating that different classes of flavonoids may induce an adaptive response.

Still, permeability coefficients of the transported flavan-3-ols (see Table 9) corresponds with previous values reported in literature (Zhang et al., 2004) and are indicative of their low oral bioavailability ($P_{app} < 2 \times 10^{-5}$) (Hubatsch et al., 2007). Since pretreatment with both extracts significantly increased TEER values, this suggests that pretreatment impacted tight junction proteins and that the observed increases in flavan-3-ols transcellular flux are not mediated by paracellular transport. An increase in tight junction proteins has similarly been observed in rodents treated with grape seed extract (Goodrich et al., 2012), which may be linked to this treatment resulting in a reduction of a marker of gut neutrophil infiltration in these animals.

Though there were no differences in intracellular methylated EGCG concentrations with pretreatment, there was a significantly decrease in formation rate of both sulfate and methyl sulfate metabolites with 10 µM pretreatment. These data suggest a inhibitory effect of flavan-3-ol pretreatment on these processes, as flavonoids have been reported to inhibit sulfotransferases (James and Ambadapadi, 2013). This is in contrast to a report of HepG2 hepatocytes pretreated for 24-48 h with 10 µM resveratrol that showed increased gene and protein expression of sulfotransferase enzymes (Lançon et al., 2007). This suggests that class of the phenolic used in treatments and length of treatment time combined with tissue type may be critical to consider in assessing their influences on Phase II metabolizing systems. Though we observed transient increases in transport with EGCG pretreatment, an animal study with a two-week pretreatment of 0.32% EGCG diet observed an approximately 50% reduction in EGCG plasma AUC (James et al., 2015). However, his study did not measure

metabolites of the compound and it is possible that enhanced metabolism could be responsible for the reduction in levels of circulating native EGCG. In contrast, a clinical study with a two week run-in period providing 800 mg EGCG per day resulted in ≈60% increase in plasma AUC (Chow et al., 2003), indicating that there may be a differential response to pretreatment depending on the specifics of the experimental design.

In contrast to EGCG pretreatment decreasing metabolizing systems, there was significantly greater concentrations of methylated EC metabolites with pretreatment at 60-90 min (Figure 10). Since 10 µM pretreatment did not significantly increase intracellular concentrations of EC but methylated EC did increase, the increase in methylated EC levels may not simply be due to increased intracellular levels of the parent compound but may in fact reflect induction of methylation systems or EC being preferentially trafficked to these systems for more efficient conjugation. However, this was not fully assessed in these experiments and remains to be investigated. In contrast, we observed a significantly lower appearance rate of EC sulfate metabolites with 10 µM pretreatment compared to control (Table 8), suggesting differential regulation of the individual Phase II systems with pretreatment. Taken together, changes in metabolism of phenolic compounds occurring in intestinal tissues with chronic exposure may shift its metabolism to other tissues such as the liver which then may then results in an altered systemic metabolite profile.

We were not able to detect glucuronide metabolites of flavan-3-ols using our model, though some (Rodriguez-Mateos et al., 2014) but not others (Sanchez-Bridge et al., 2015) have been able to detect this metabolite. This may be related to differences in

analytical methods used in addition to varying cell culture conditions, treatment concentrations, and length of treatment times. In addition, we did not observe significant levels of metabolites in the three compartment model. Again, this may be related to our use of extracts rather than isolated compounds, which may provide additional substrate that saturate the metabolizing systems (Sanchez-Bridge et al., 2015). In comparing our results to *in vivo* models, we did not observe a similar magnitude of effects in the adaptive response in our cell model as compared to animal studies previously cited (Ferruzzi et al., 2009; Wang et al., 2012). This may be due, in part, to differences in the pretreatment concentrations used in the studies and the actual concentrations employed in the animal studies. Luminal concentrations in the cited animal studies are estimated to be in the order of ≈4.3 mM concentrations of monomeric (epi)catechins (Kararli, 1995), in contrast to the lower pretreatment concentrations used in our study (1-10 µM). Thus our experimental paradigm may be more indicative of modest dietary consumption of flavan-3-ols, while the animal study represents what may be occurring with exposure to amounts common in dietary supplements. Though luminal concentration of flavonoids could potentially be higher from typical servings of foods (Scalbert and Williamson, 2000), relatively low doses were chosen here were based on preliminary screening of cytotoxicity and to mimic more modest concentrations of flavan-3-ols achieved by typical consumption in the U.S. (Kim et al., 2014). Another point to consider is that though we did not quantify proanthocyanidins in the grape seed extract, they appear to be an important

component in the observed adaptive response in animal models that occurs with chronic exposure to C/EC monomers and polymers.

Since differentiation of intestinal cells is influenced by its matrix (Reya and Clevers, 2005), it is plausible that altering that environment will affect the differentiation process. In support of this, Caco-2 cells chronically treated with flavan-3-ol-rich grape seed and other grape products over ≈30 d exhibit differences in expression of classic markers of differentiation such as sucrase-isomaltase and aminopeptidase N (Laurent et al., 2004, 2005). Though the present experiments did not measure markers of differentiation, we did observe increases in TEER values with treatment, which may indicate altered differentiation. Also, other tissue types appear to respond to pretreatment as primary hepatocytes have altered gene expression of organic anion transporting polypeptides (OATPs) with long-term exposure to select anthocyanins (Riha et al., 2015). As these markers were not followed specifically in this study, future work will be required to identify specific proteins that may be altered with pretreatment such as efflux transporters, which have been reported to be affected by the phenolic acid caffeic acid (Hong et al., 2015).

A limitation of this model is that it does not consider other tissues involved in absorption and transport of flavonoids, such as the liver and kidney. In addition, it only focuses on adaptation occurring in the upper gastrointestinal tract, which does not incorporate changes in gut microbiota in the lower bowel that may occur with a flavonoid-rich diet. Ultimately, it is important to consider absorption and metabolism of these compounds in the context of a diet, where there can be multiple interactions

between food components and individual flavonoids that can alter metabolism of other compounds (Zhang et al., 2004; Zhu et al., 2008). Still, these results suggest that intestinal adaptation to chronic exposure to both isolated flavan-3-ols and extracts rich in flavan-3-ols can alters flavan-3-ol transport and potentially intestinal barrier function.

3.5 Conclusion

Our data suggest pretreatment with isolated flavan-3-ols and extracts rich in these compounds are both able to elicit an adaptive response in the Caco-2 cell model that is directionally reflective of in vivo adaptations previously documented. Since this model is widely used to predict the bioavailability of phytochemicals, the experimental paradigm of chronic exposure could possibly allow for more accurate assessment of the absorption and metabolism of these compounds from exposure due to broad dietary patterns. In addition to the increased transport of green tea and grape seed flavan-3-ols observed with pretreatment, differences in EGCG and EC metabolite profile with pretreatment were observed. These data compare to previous pre-clinical and clinical studies demonstrating adaptation with chronic exposure to these compounds. Though not directly assessed in these experiments, increased flux of flavan-3-ols observed after pretreatment suggests induction of more efficient cellular trafficking of the compounds. More complex models incorporating aspects of Phase II metabolism other than intestinal metabolism may assist in elucidating alterations occurring at other tissue sites. In addition, future studies can further explore this question by determining molecular mechanisms involved in this adaptive response. Taken together, these results

demonstrate that the Caco-2 cell model may be used model alterations in intestinal transport and metabolism that occur with chronic dietary exposure to select flanvan-3-ols.

CHAPTER 4. ADAPTATION IN CACO-2 HUMAN INTESTINAL CELL DIFFERENTIATION AND PHENOLIC TRANSPORT WITH CHRONIC EXPOSURE TO PHENOLIC-RICH BLACKBERRY (RUBUS SP.) EXTRACT

As part of the manuscript in review in Molecular Nutrition & Food Research, BW Redan[1], GP Albaugh[2], CS Charron[2], JA Novotny[2], and MG Ferruzzi,[1,3]

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4.1 Introduction

Berries are a rich dietary source of bioactive phenolic compounds including anthocyanins, phenolic acids, flavonols, and flavan-3-ols. Though consensus has not been reached (Jacques et al., 2015), data from epidemiological studies conducted in various populations support the notion that diets high in fruits, including berries, are associated with a decreased risk of chronic disease and overall mortality (Du et al., 2016; Hjartåker et al., 2015). In addition, data from clinical studies have shown that consumption of dietary sources of anthocyanins and other flavonoids from berries can improve risk factors for cardiovascular disease such as blood pressure and lipid profiles (Kianbakht et al., 2014; Novotny et al., 2015). As such, blackberry (Rubus sp.) was selected for these studies since it is rich in the anthocyanin cyanidin-3-*O*-glucoside, the

aglycone of which is the most common in the food supply and most highly consumed anthocyanin form (Wu et al., 2006). Blackberries contain primarily cyanidin-3-*O*-glucoside and do not contain as wide array of anthocyanins, including more structurally complex acylated forms found in many other berries (Kaume et al., 2012; Wu and Prior, 2005).

Still, translation of such findings to dietary strategies to prevent chronic diseases is limited by several factors including the poor oral bioavailability of flavonoid compounds from food. Excluding microbial metabolites, less than 5% of a single acute oral, food-based dose of flavonoids are commonly reported to be absorbed (Neilson and Ferruzzi, 2011), though higher bioavailability values have been reported for ingestion of purified flavonoids (Manach et al., 2005). However, bioavailability from single oral doses, the most typically applied assessment of oral bioavailability, may not be indicative of the more common dietary paradigm of repeated daily exposure encountered by high consumers of fruits and vegetables. Evidence does exist that long-term exposure to flavonoids can significantly alter bioavailability (Chow et al., 2003; Ferruzzi et al., 2009) and metabolism of flavonoids (Wang et al., 2015, 2012). In an animal study, 10 day administration of a grape seed extract improved plasma response of catechin (C)/epicatechin (EC) (Ferruzzi et al., 2009) and C/EC glucuronides and methyl-glucuronides (Wang et al., 2012). In addition, clinical work has demonstrated that 4 week chronic daily administration of EGCG increased its AUC by approximately 60% (Chow et al., 2003).

While an adaptive response to phenolic chronic exposure has been observed in both pre-clinical and clinical models, the mechanisms behind this phenomenon are not well understood. Since dietary patterns typically result in a paradigm of chronic exposure to various phenolic compounds, there is a need to better understand the adaptive phenomena so they can be leveraged to improve bioavailability and delivery of key phenolic metabolites related to disease-preventative endpoints. Considering the gastrointestinal tract is the main barrier to delivering dietary phenolics systemically, we hypothesize that adaptation occurring in the upper gastrointestinal epithelium is a key factor modifying absorption of phenolics and may be responsible, in part, for the observed differences in flavonoid absorption and metabolism in chronic dosing paradigms.

To gain a better mechanistic understanding of bioavailability, researchers have utilized differentiated Caco-2 cell monolayers to study transport of pharmaceuticals and phytochemicals since they are highly predictive of human absorption (Sambuy et al., 2005). However, most studies involving the Caco-2 cell model are limited to acute experimental paradigms and do not consider how chronic exposure typical of dietary patterns modulates physiological activities, such as absorption at the gut epithelium. Since the Caco-2 model has exhibited differential transport of phenolics in acute versus a long-term treatment with grape-derived anthocyanins (Faria et al., 2009), we proposed simulating the chronic treatment paradigm from previous animal studies using Caco-2 cell monolayers to determine if we could model an adaptive response in transport of blackberry phenolics.

4.2 Materials and Methods

4.2.1 Preparation of Blackberry extract

Composite blackberry extract was produced from frozen blackberries, which were a combination of Rubus laciniatus, Rubus Marion, and Rubus fruticosus L. purchased from SYSCO (Houston, TX). Blackberries were freeze-dried and then extracted with methanol:H2O (80:20; v:v) and resulting extracts dried under a nitrogen stream. Bulk extract was then resoluabilized in H2O:HCl (99.9:0.01; v:v) and purified using an Extract Clean™ 10,000 mg/75 mL C18-HC column (Grace, Columbia, MD). Briefly, the column was activated with 1 column volume (75 mL) MeOH, followed by 3 column volumes H2O. Blackberry extract was loaded onto the column and successively washed with 4 column volumes H2O to remove ascorbic acid, sugars, and other osmotically active compounds, dried for 30 s with vacuum, and then eluted with 1 column volume of MeOH:HCl (99.9:0.01; v:v). The eluate was then aliquoted into glass tubes, dried under a nitrogen stream, and stored at -80°C until further use.

4.2.2 Cell culture and treatments

Caco-2 TC7 cell line (passages 81-84) was maintained in DMEM media with 10% FBS with 10% v/v FBS, 1% v/v nonessential amino acids, 1% v/v HEPES, 1% v/v penicillin/streptomycin, and 0.1% v/v gentamicin. Cells were seeded at a density of 2.12 x 10^5 cells/cm^2 on a Transwell® insert system (Corning® polyester membrane, 24 mm diameter, pore size 0.4 µm), and monolayers were differentiated over a period of approximately 25 d post-confluency. Total phenolic concentration of blackberry extract

was determined by the Folin-Ciocalteu total phenolic method (Waterhouse, 2001) and sterile filtered (0.2 µm filter). Cells were cultured in growth media with a final concentration of either 0 µM (control), 1 µM, or 10 µM blackberry phenolics, added to the apical chamber (see **Figure 13** for outline of experimental design). Preliminary experiments showed that cell monolayers could be chronically treated with 10 µM of extract without significantly decreasing cell viability (>95%) as determined by MTT assay (Biotium, Hayward, CA). All growth media was changed every 48 h.

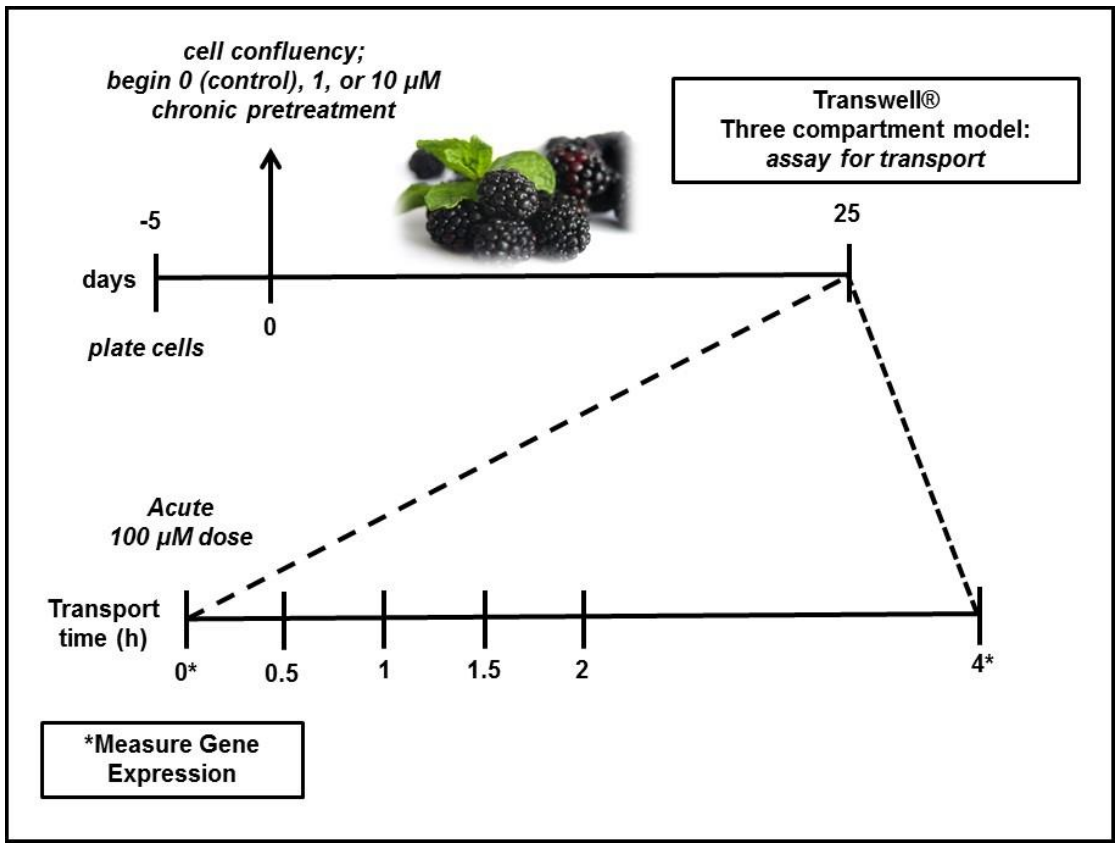


Figure 13. Experimental design to simulate chronic treatment of Caco-2 cell monolayers. Cells were cultured in growth media with either 0 µM (control), 1 µM, or 10 µM of blackberry extract added to the apical chamber. After all cells were cultured in control media for 24 h, uptake was assessed over 4 h with a 100 µM acute dose of blackberry extract.

4.2.3 Transport of Phenolic Compounds

Before assessing transport of phenolics, cell monlayers were cultured for 24 h in control growth media, rinsed twice with 0.1% fatty-acid free albumin in PBS, and followed with PBS only. Transport of blackberry extract was assessed using an acute dose of 100 µM total phenolics in PBS transport buffer (pH = 5.5). 1.5 mL transport media was added to the apical chamber and 2 mL of PBS (pH = 5.5) was initially placed in the basolateral chamber, after which 1 mL PBS was collected and replaced with fresh PBS at 0 (immediately collected after transport media was loaded), 30, 60, 90, 120, and 240 min. Experiments were performed in quadruplicate. After the 4 h uptake period, cells were rinsed according to the above procedure and then collected in ice cold PBS (pH = 5.5). Transport media was acidified with 1% v/v formic acid/acetonitrile and then left on ice for 10 min to precipitate protein, and centrifuged at 18,000 *g* for 5 min to remove particulates before injecting 20µL in the LC-MS-TOF system. Stability of compounds in transport media over 4 h was assessed by incubating cell-free transport media in 6-well plates, and recovery of cyanidin-3-*O*-glucoside in the media was found to be 98.1 ± 3.75% (mean ± SEM).

4.2.4 Analysis of Blackberry Extract and Test/Transport Media

Analysis of blackberry extracts and test/transport media was adapted from methods previously described (Song et al., 2013, 2015). Briefly, separations of phenolics was achieved using a Waters RP-C18 column (Xbridge BEH Shield, 2.5 µm, 2.1x100 mm) and characterized using an Agilent 1100 high-pressure liquid chromatography system in

tandem with a Waters LCT Premier time-of-flight mass spectrometer (LC-TOF-MS) with electrospray ionization (ESI) in either positive (for anthocyanins) or negative mode (for other phenolics). The flow was set to 0.25 mL/min and mobile phases were A: 2% formic acid (aq.); B: acetonitrile (0.1% formic acid) for anthocyanin analysis and A: 0.4% formic acid (aq.); B: acetonitrile (0.1% formic acid) for other phenolics. A linear gradient condition for eluting anthocyanins was as follows: initial conditions were 5% B; 0-15 min: 5-25% B; 15-18 min: 25-30% B; 18-19 min: 30-5% B; 19-23.5 min: hold at 5% B to reset the gradient. Conditions for other phenolics was 5% B at 0 min; 0-15 min: 5%-35% B; 15-17 min: 35-70% B; 17-19 min: 70-5% B; hold at 5% B at 23 min to reset the gradient. Mass-to-charge (*m/z*) ratios used to produce extracted ion chromatograms (EICs) and used to characterize and quantify compounds are displayed in **Table 10**. Cyanidin-3-*O*-glucoside was characterized using MS and then quantified using photodiode array (PDA) response at 520 nm. Standard curves using pure standards of cyanidin-3-*O*-glucoside (Chromadex, Irvine, CA), (+)-catechin, (-)-epicatechin, quercetin-3-*O*-glucoside, kaempferol-7-*O*-glucoside, and 3-*O*-caffeoylquinic acid (Sigma, St. Louis, MO) were produced to quantify transported phenolics.

4.2.5 Gene expression

In order to determine alteration of xenobiotic transport and metabolizing system gene expression due to chronic exposure, a parallel set of Caco-2 cell experiments was conducted with cells treated as previously described in Section 2.2. RNA was isolated using RNeasy spin columns according to the manufacturer's directions (Qiagen Sciences,

Germantown, MD). RNA was analyzed by Experion RNA gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) for its quality and quantity, reverse transcribed to cDNA, and then plated onto the Drug Metabolism: Phase II Enzymes PCR Array (SABiosciences, Germantown, MD, catalogue no. PAHS-069Z) and the Drug Transporters PCR Array (SABiosciences, Germantown, MD, catalogue no. PAHS-070Z), following manufacturer's instructions to determine alterations in expression of the respective genes. Real-time PCR was conducted using a CFX96 real-time PCR system (Bio-Rad laboratories).

4.2.6 Data Analysis

Values for phenolics are expressed as average pmol per well ± SEM (n = 4). The apparent permeability coefficient (P_{app}) was calculated using an equation previously reported using the Caco-2 cell model to measure transport of compounds through the cell monolayer (Hubatsch et al., 2007). Gene expression values are displayed as average (n = 3) $\Delta\Delta C_t$ fold-changes in mRNA expression relative to housekeeping genes. Displayed data were selected by determining genes with significant ($P < 0.05$) or near significant changes in expression. Analysis of gene expression data was performed using an Excel (Microsoft, Redmond, WA) spreadsheet template as provided by SABiosciences. All other analyses were performed using SAS 9.3 (SAS Institute, Cary, NC), and significant differences were evaluated by a Bonferroni post-hoc pairwise test ($\alpha = 0.05$).

4.3 Results

4.3.1 Differential transport of blackberry phenolics with pretreatment

Phenolic composition of the extract is shown in **Table 11**. The main anthocyanin in the blackberry extract was cyanidin-3-*O*-glucoside, which corresponds to previously reported values (Kaume et al., 2012; Mertz et al., 2007). Interestingly, this blackberry preparation did have greater amounts of kaemperfol glycosides and less quercetin glucoside compared to previous reports (Mertz et al., 2007), but this may simply be due to differences between geographic origin, harvest season and specific variety. The molecular ion corresponding to 515 *m/z* was tentatively identified as dicaffeoylquinic acid.

Table 10. Mass-to-charge (*m/z*) ratios and ionization mode used to characterize phenolic compounds in treatment and transport media using LC-TOF-MS.

Compound	m/z	Ionization mode
Cyanidin-3-*O*-glucoside	449	+
Catechin/Epicatechin	289	−
Kaempferol glucoside	447	−
Kaempferol glycoside derivative	465	−
Quercetin glucoside	463	−
Dicaffeoylquinic acid	515	−

Since major phase II metabolites of the parent compounds found in the blackberry extract were not observed to a great extent in this model (data not shown), we focused on tracking the parent compounds for flux through the cell monolayers. **Figure 14** displays cumulative apical to basolateral transport data for the seven major phenolic species in the blackberry transport media. After 2 h, 1 µM pretreated monolayers showed significant ($P < 0.05$) differences in transport including a 34% decrease in cumulative transport of cyanidin-3-*O*-glucoside compared to control (0 µM pretreatment) (7.14 vs. 4.75 nmol; 0 µM vs. 1 µM pretreatment) and 30% less transport of dicaffeoylquinic acid (6.61 vs. 4.64 nmol; 0 µM vs. 1 µM pretreatment). After 4 h, 1 µM pretreated monolayers were observed to have reduced transport of every phenolic

compound other than cyanidin-3-*O*-glucoside including 33% less catechin (0.979 vs. 0.654 nmol; 0 µM vs. 1 µM pretreatment), 42% less epicatechin (2.48 vs. 1.43 nmol; 0 µM vs. 1 µM pretreatment), 19% less kaempferol glucoside (41.1 vs. 31.5 nmol; 0 µM vs. 1 µM pretreatment), 23% kaempferol glycoside derivative (17.5 vs. 14.1; 0 µM vs. 1 µM pretreatment), 20% less quercetin glucoside (1.90 vs. 1.19; 0 µM vs. 1 µM pretreatment), and 30% less dicaffeoylquinic acid (20.9 vs. 14.2; 0 µM vs. 1 µM pretreatment). While there were no significant differences in phenolic transport over 4 h with 10 µM pretreatment, it significantly ($P < 0.05$) exhibited decreased transport of kaempferol glucoside (15.2 vs. 12.2 nmol; 0 µM vs. 10 µM pretreatment) and dicaffeoylquinic acid (6.61 vs. 4.60; 0 µM vs. 10 µM pretreatment) over 2 h. In addition, a decrease in quercetin glucoside transport was observed after 90 min in the 10 µM pretreated monolayers compared to 10 µM pretreatment (0.380 vs. 0.197 nmol).

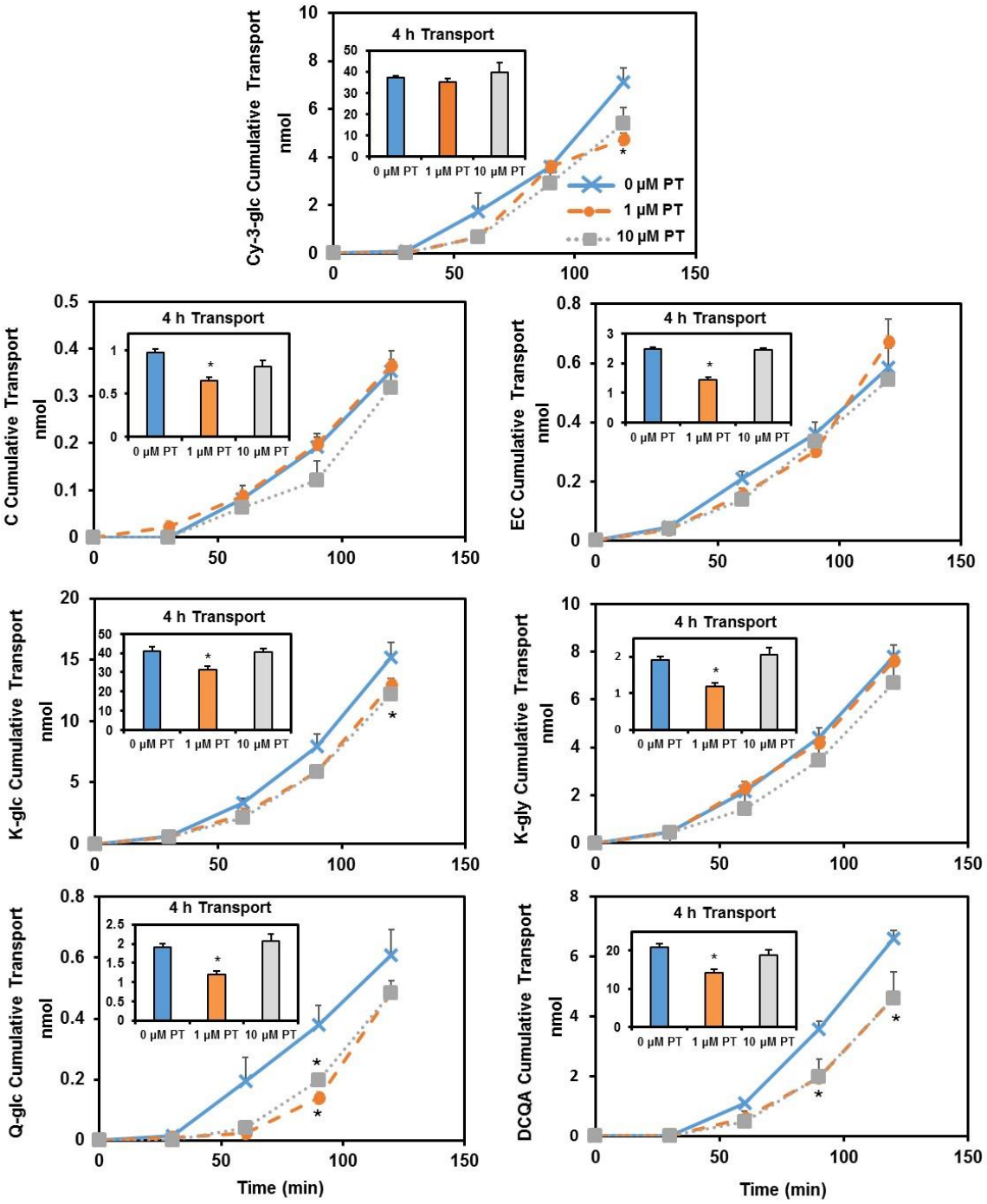


Figure 14. Chronic pretreatment with blackberry fruit phenolics differentially alters apical to basolateral transport of blackberry fruit phenolics across differentiated Caco-2 cell monolayers. Confluent Caco-2 monolayers were cultured on Transwell® inserts in pretreatment (PT) media containing either 0 (control), 1, or 10 µM total blackberry phenolics (see table 2 for composition of blackberry transport media) during the differentiation process. All cell monolayers were incubated with control media 24 h

(Figure 14 caption, con'd) before transport was assessed using a 100 µM acute dose of total blackberry phenolics over 4 h. Left panel displays uptake data over 2 h while right panel is over 4 h. Statistical pairwise comparison was performed comparing 0 (control) to 1 or 10 µM PT. *$P < 0.05$ compared to control. Abbreviations: PT, pretreatment; Cy-3-glc, cyanidin-3-O-glucoside; C, catechin; EC, epicatechin; K-glc, kaempferol glucoside; K-gly, kaempferol glycoside derivative; Q-glc, quercetin glucoside; DCQA, dicaffeoyquinic acid.

Table 11. Blackberry Transport Loading Media phenolic Composition (100 µM total phenolics)[a]

Phenolic class	Compound	µM ± SD
Anthocyanin	Cyanidin-3-*O*-glucoside	37.70 ± 1.05
Flavan-3-ols	Catechin	0.230 ± 0.0213
	Epicatechin	3.339 ± 0.107
Flavonols	Kaempferol glucoside	13.119 ± 0.779
	Kaempferol glycoside derivative	8.314 ± 0.258
	Quercetin glucoside	2.34 ± 0.14
Phenolic acid	Dicaffeoylquinic acid	19.80 ± 0.255
	TOTAL	84.73

a) Values shown are displayed as average ± SD (n = 3).

Table 12. Apparent permeability coefficients (P_{app}) of blackberry phenolics across differentiated Caco-2 cell monolayers.[a]

Compound	Pretreatment	log P_{app} (cm/s) over 2 h	log P_{app} (cm/s) over 4 h
Cyanidin-3-O-glucoside	0 μM	-7.26 ± 0.855	-6.82 ± 0.846
	1 μM	-7.38 ± 0.875**	-6.85 ± 0.820
	10 μM	-7.36 ± 0.856*	-6.77 ± 0.778
Catechin	0 μM	-6.33 ± 0.740	-6.18 ± 0.761
	1 μM	-6.33 ± 0.744	-6.34 ± 0.760**
	10 μM	-6.36 ± 0.713	-6.25 ± 0.726[#]
Epicatechin	0 μM	-6.97 ± 0.808	-6.77 ± 0.802
	1 μM	-7.00 ± 0.837	-6.91 ± 0.816**
	10 μM	-7.03 ± 0.773	-6.79 ± 0.801
Kaempferol glucoside	0 μM	-6.44 ± 0.761	-6.31 ± 0.757
	1 μM	-6.55 ± 0.790*	-6.42 ± 0.772*
	10 μM	-6.57 ± 0.774*	-6.32 ± 0.765
Kaempferol glycoside derivative	0 μM	-6.55 ± 0.771	-6.48 ± 0.785
	1 μM	-6.57 ± 0.799	-6.57 ± 0.785*
	10 μM	-6.63 ± 0.805*	-6.49 ± 0.799
Quercetin glucoside	0 μM	-7.10 ± 0.794	-7.30 ± 0.754
	1 μM	-7.64 ± 0.787*	-7.49 ± 0.775
	10 μM	-7.64 ± 0.791*	-7.25 ± 0.749
Dicaffeoyquinic acid	0 μM	-6.97 ± 0.783	-6.78 ± 0.801
	1 μM	-7.17 ± 0.831*	-6.92 ± 0.801*
	10 μM	-7.17 ± 0.857*	-6.83 ± 0.811

Table 12 legend.

a) Values are presented as average (n = 4) ± SEM. Statistical pairwise comparison is between pretreatments and 0 µM.

*P<0.05; **P<0.01; $^{\#}P$=0.0503.

Iterestingly, phenolics were not detectable in cell monolayers after 4 h exposure to any treatment.

Table 12 displays transport data as log apparent permeability (P_{app}) calculated from the rate of each phenolic's transcellular flux. While log P_{app} of cyanidin-3-O-glucoside was not affected over 4 h, both pretreatment conditions significantly (P < 0.05) decreased its log P_{app} over 2 h from -7.26 cm/s for 0 µM pretreated to -7.38 and -7.36 cm/s for 1 and 10 µM pretreatment, respectively. For flavan-3-ols catechin and epicatechin, log P_{app} significantly decreased over 4 h and not 2 h for the 1 µM pretreatment and reached near significance (P = 0.0503) for catechin transport over 4 h. For flavonols, kampferol glucoside showed decreased log P_{app} for both treatments over 2 h but only 1 µM treatment over 4 h, and similar results were found for the kaempferol glycoside derivative. Quercetin glucoside exhibited significantly decreased log P_{app} for both treatments at 2 h but not 4 h. Lastly, dicaffeoyquinic acid showed decreased log P_{app} for both treatments over 2 h but only 1 µM pretreatment reached statistical significance over 4 h.

4.3.2 Differential changes in mRNA expression of xenobiotic transport and metabolizing systems

Figure 15 displays alterations in expression of xenobiotic metabolizing system genes with 4 h 100 µM acute treatment only and 10 µM pretreatment plus 100 µM acute treatment compared to differentiated cell monolayers. These data show significant ($P < 0.05$) alterations in gene expression related to Phase II metabolism including methylation, sulfation, and glucuronidation for both treatments. However, six out of the 15 genes displayed in Figure 15 were significantly ($P < 0.05$) different only with pretreatment in contrast to cell monolayers solely receiving acute treatment. The results showed increased expression of one of the three genes UGTs (*UGT2B10*), while there was decreased expression of both *UGT1A4* and *UGT1A8*. Gene expression was altered in four SULT genes, with pretreatment alone resulting in a significant decrease in *SULT2A1* expression. *SULT4A1* expression decreased to a greater magnitude with pretreatment (-4.5 fold) compared to acute treatment only (-1.6 fold). Three methylation genes (*GNMT*, *HNMT*, and *TPMT*) were significantly altered from the treatments, but *GNMT* was only altered with pretreatment.

Figure 16 displays transport genes whose expression was impacted by both the acute and chronic treatments. Eight out of the 15 transporters displayed were only significantly ($P < 0.05$) affected by pretreatment plus acute treatment. Overall, there was decreased expression of transport systems except *SLC7A11*, which significantly increased by almost four fold with pretreatment but not acute treatment.

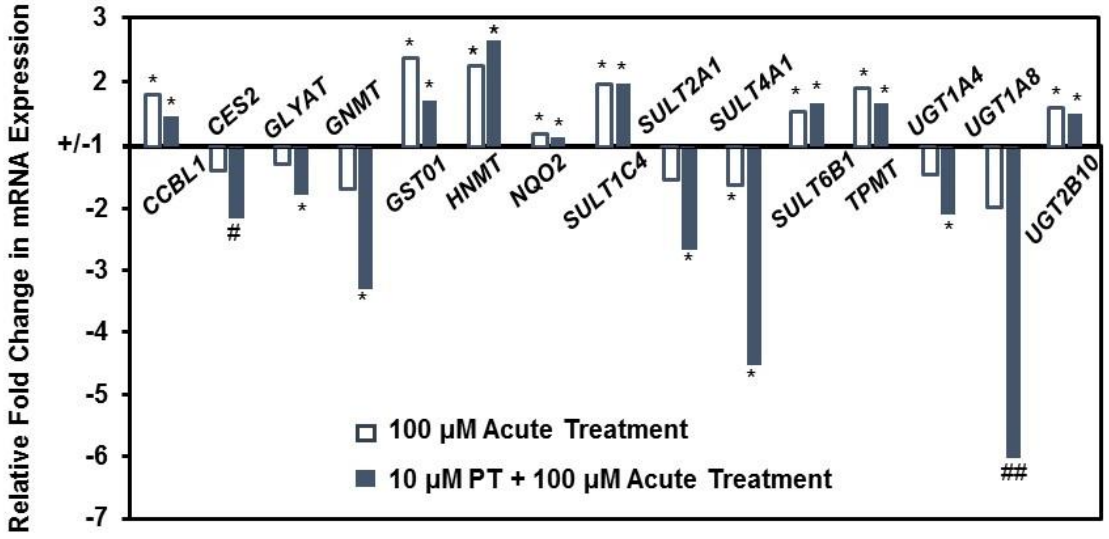


Figure 15. Differential alteration in gene expression of Phase II metabolizing enzymes in differentiated Caco-2 cells due to chronic pretreatment (PT) compared to acute only treatment with blackberry fruit extract. Values are displayed as average (n = 3) $\Delta\Delta C_t$ fold-changes in mRNA expression relative to selected housekeeping genes. Statistical pairwise comparison was performed comparing differentiated Caco-2 cells to treatments. $^{*}P < 0.05$ compared to control; $^{\#}P = 0.056$ compared to control; $^{\#\#}P = 0.0501$.

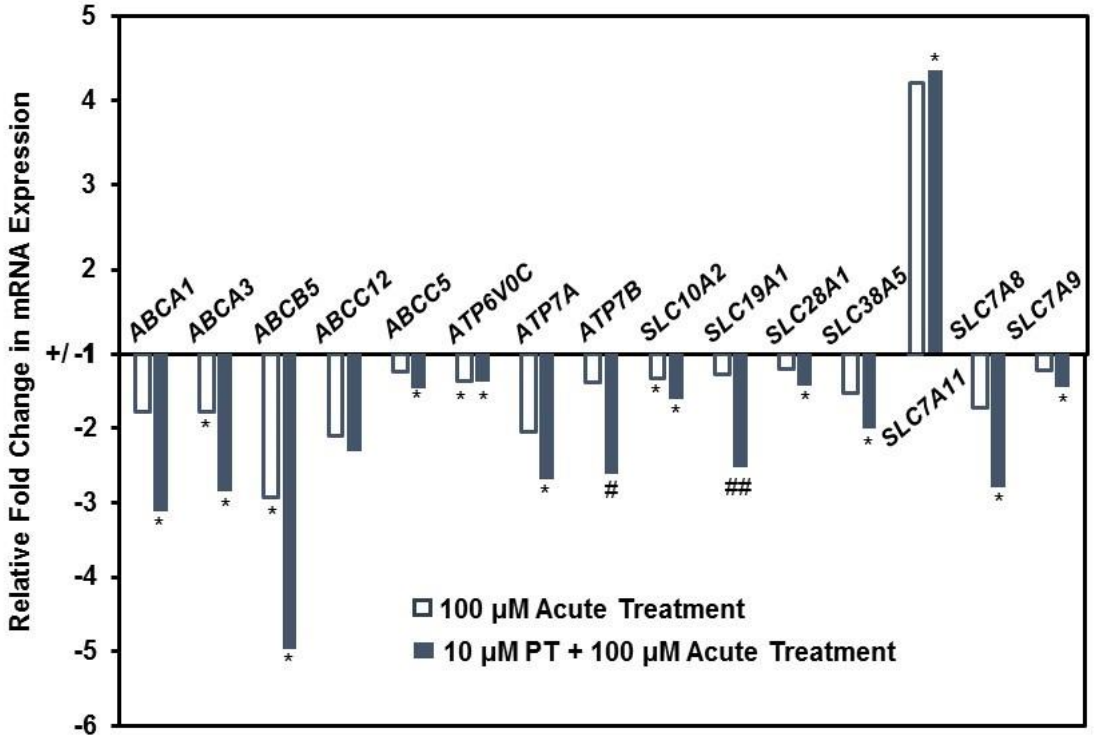


Figure 16. Differential alteration in gene expression of transport systems in differentiated Caco-2 cells due to chronic pretreatment compared to acute only treatment with blackberry fruit extract. Values are displayed as average (n = 3) $\Delta\Delta C_t$ fold-changes in mRNA expression relative to selected housekeeping genes. Statistical pairwise comparison was performed comparing differentiated Caco-2 cells to treatments. *$P < 0.05$ compared to control; $^{\#}P = 0.063$; $^{\#\#}P = 0.0687$.

4.4 Discussion

The Caco-2 cell model has been previously used to elucidate mechanism of absorption and metabolism for phenolics connected by epidemiological associations between berry consumption and reduced risk of chronic disease. Caco-2 experiments have demonstrated that blackberries and other berries rich in anthocyanins have been

characterized to have effects including decreased peroxyl radical production (Elisia and Kitts, 2008) to decreased gene expression of GLUT2 and SGLT1 (Alzaid et al., 2013). In addition, the three compartment Caco-2 cell model used in this study has been widely utilized to assess intestinal transport of flavonoids and phenolic acids from various berry extracts and has also been done in parallel to clinical studies (Kuntz et al., 2015). However, characterization of the effect of chronic exposures that mimic dietary patters rich in berry foods on phenolic transport and metabolism is limited. Since intestinal enterocytes have a high turnover rate and the nascent cells repopulating the epithelial lining are influenced by the extracellular matrix (Reya and Clevers, 2005), it is possible that dietary factors including phenolics can influence the differentiating cell's function. Supporting this notion are data demonstrating that Caco-2 monolayers chronically treated with extracts of grape seed and grape products during the differentiation process exhibit altered markers of differentiation such as sucrase-isomaltase and aminopeptidase N (Laurent et al., 2004, 2005), suggesting that the addition of phenolic-rich extracts to cellular media is fundamentally altering the cell's phenotype.

Our data overall show significant ($P < 0.05$) decreases in the cumulative transport of phenolic compounds across Caco-2 cell monolayers with pretreatment of berry extracts in a fashion that mimics the exposure of a chronic dietary pattern rich in berries. This is in contrast to Faria *et al.* which showed an approximately 50% increase in transport of anthocyanins with 96 h pretreatment of phenolic-rich grape skin extract before assessing transport in Caco-2 cells (Faria et al., 2009). Additionally, these results differ from animal work done with grape seed extract rich in flavan-3-ols (Ferruzzi et al.,

2009; Wang et al., 2012) and a human study that administered isolated EGCG (Chow et al., 2003), which both reported increased bioavailability with chronic exposure. There are likely several reasons for these differences, one being that Faria *et al.* pretreated Caco-2 monolayers with ≈400 µM phenolics (200 µg/mL grape skin anthocyanins), and the animal and clinical study would have produced concentrations in the gut lumen estimated to be ≈4.3 mM and ≈900 µM, respectively. While it is possible that luminal concentration of flavonoids would be higher from typical servings of foods (Scalbert and Williamson, 2000), relatively low doses were chosen here (1-10 µM) based on preliminary screening of cytotoxicity and to mimic more modest concentrations achieved by typical consumption of fruits in the U.S. (approximately 100 g per day) for both children (Kim et al., 2014) and adults (Kimmons et al., 2009). Thus, use of these relatively lower concentrations in the current study may partially explain the differences in the directionality of the observed changes in transport between animal studies and preclinical cell-based studies here.

Other differences between this study and others assessing phenolic transport include our use of anthocyanin-rich blackberry extract, which contains a different milieu of phenolic compounds than those used previously, suggesting that intestinal adaptation may be dependent on phenolic class and matrix. Additionally, since Caco-2 is a human intestinal cell line, it is not unexpected for the data to be somewhat different from animal models. Another consideration for our results is that phenolic extracts were used in order to isolate the effect of phenolic compounds over non-phenolic compounds

such as ascorbic acid and sugars, which have both been reported to increase bioavailability of flavan-3-ols (Peters et al., 2010).

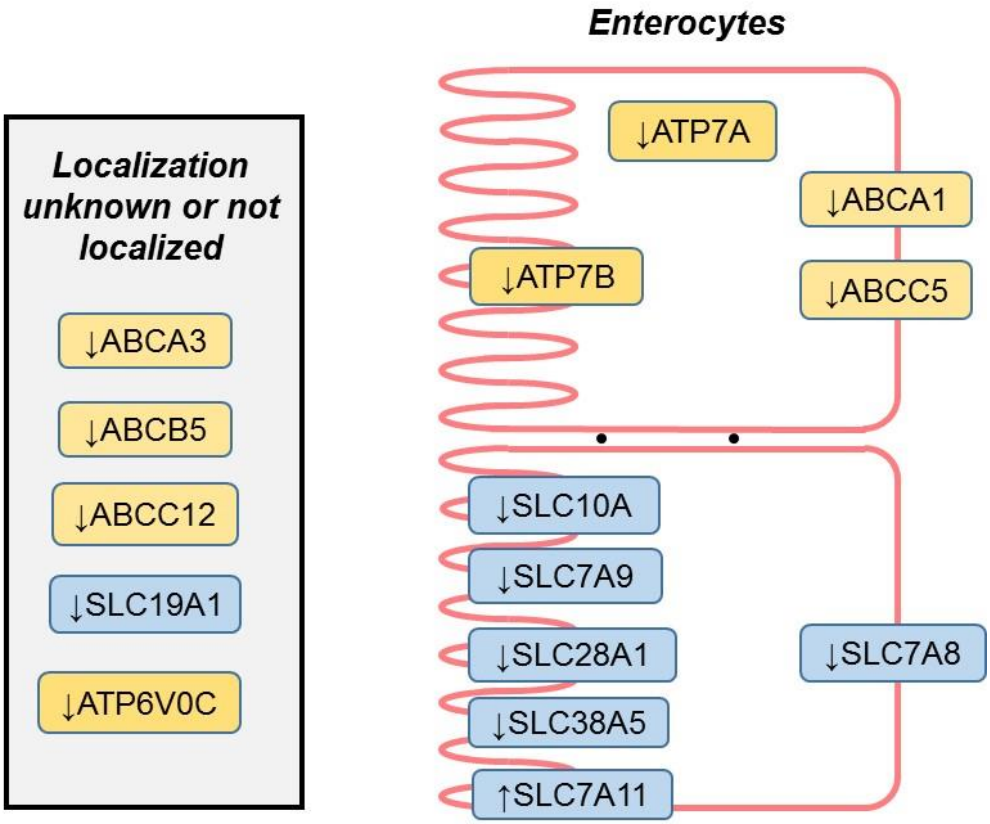


Figure 17. Enterocyte localization of transport proteins affected by blackberry extract treatments and directional change of its mRNA expression.

There appeared to be greater alteration in transport over 4 h from 1 µM but not 10 µM pretreated monolayers (see Figure 16), which demonstrates that it is critical to assess transport over a range of time points. The decreases in log P_{app} values with

chronic exposure (Table 12) indicates that there may be less absorption of these compounds in vivo with pretreatment since log P_{app} values have been reported to correlate with the fractional absorption of select pharmaceutical compounds in humans (Hubatsch et al., 2007). As such, this decrease in log P_{app} values may therefore indicate decreased intestinal transport with chronic exposure in a clinical setting. Since phenolics were not detectable in cell monolayers in any treatment group, differences in transport are likely not due to increased cellular accumulation.

Our data showed differences in gene expression of the Phase II metabolizing enzymes with blackberry exposure. We observed significant ($P < 0.05$) decreases in mRNA expression of Phase II metabolizing enzymes *UGT1A4* and borderline significant ($P = 0.0501$) decreased expression of *UGT1A8* with pretreatment (see Figure 15). This decrease may be due to the large concentration of glycosylated compounds (for example, anthocyanin and flavonol glycosides) present in blackberry extract, since phenolic glycosides have been reported to reduce circulating glucuronidated metabolites in rodent models and decrease gene expression of *UGT1A8* in rat intestinal tissue (Ma et al., 2013). As such, decreasing glucuronidation systems in the intestine may shift xenobiotic metabolism to other tissues such as the liver and kidney. Still, mRNA expression of other Phase II metabolizing enzymes show increased expression (such as sulfotransferases and methylation enzymes) apparently due to the inducible nature of these systems. Though Phase II metabolites of the flavan-3-ols have been reported in Caco-2 cell model (Rodriguez-Mateos et al., 2014; Zhang et al., 2004), we were unable to detect metabolites, which may be related to their concentrations being

under the limit of detection of our instrumentation or due to the use of complex plant extracts rather than isolated phenolics used in previous studies.

Overall, gene expression of transport systems decreased, except for the amino acid transporter *SLC7A11*, which increased by more than four-fold (Figure 16). These decreases in gene expression may partially account for the observed lower cumulative phenolic transport. Though GLUT2 (*SLC2A2*) and SGLT1 (*SLC5A1*) have been shown to be critical to the absorption of cyanidin-3-*O*-glucoside in the Caco-2 model (Zou et al., 2014), these transporters were not significantly affected by treatments and may explain why transport of this compound was only modestly affected. In addition, gene expression was only measured after the 4 h acute treatment period, which may have not captured any differences occurring before this time interval. Localization of these transporters is also an important factor to consider (see **Figure 17**) in addition to molecular interaction of transporters with phenolic compounds, which may not necessarily be accounted for by gene expression data.

Interestingly, transepithelial electrical resistance (TEER) values significantly ($P < 0.05$) increased in 10 µM but not 1 µM pretreated monolayers (**Table 13**), which may indicate that gut barrier function may be altered through pretreatment by increasing tight junction proteins. Since the TEER values in 1 µM pretreated monolayers were not significantly different ($P > 0.05$) from control, observed differences in transport are likely due to changes in transport-mediated and not paracellular mechanisms. Apple extracts have been reported to increase TEER values in Caco-2 cell monolayers (Vreeburg et al., 2012), along with quercetin (Amasheh et al., 2008).and naringenin (Noda et al., 2013),

which both upregulate gap junction proteins. In addition to cell culture studies, this increase in levels of tight junction proteins has similarly been shown in animal models treated with flavan-3-ol rich grape seed extract (Goodrich et al., 2012).

Table 13. Background-subtracted transepithelial electrical resistance (TEER) values of differentiated Caco-2 cell monolayers used in transport experiments.[a]

Pretreatment Condition	TEER ($\Omega \cdot cm^2$)
0 µM	240 ± 4.74[a]
1 µM	240 ± 7.46[a]
10 µM	256 ± 4.37[b]

a) Values are presented as average (n = 4) ± SEM, and those with different letters are significantly different ($P < 0.05$).

One limitation of this model is that it does not consider other tissues involved in transport and metabolism of phenolics, such as the liver. Primary hepatocytes exhibit altered expression of organic anion transporting polypeptides (OATPs) with longer-term exposure (24 h) to select isolated anthocyanins (Riha et al., 2015), indicating that this tissue needs to be considered for future work. In addition, the Caco-2 model only focuses on adaptation occurring in the upper gut, which does not consider alterations in gut microbiota that may occur with a diet high in phenolics. Berries have been shown to

influence gut microbiota in animal (Jakobsdottir et al., 2013; Lacombe et al., 2013) and human (Vendrame et al., 2011) studies. Further, the gut microbiome has been reportedly modified by repeated exposure to phenolic-rich grape seed extract, leading to increased production of low-molecular weight phenolic catabolites with potential bioactive effects (Wang et al., 2015).

4.5 Conclusion

Our data show there was an adaptive response in the Caco-2 cell model following a pretreatment period, demonstrating this model is dynamic and may be used for mechanistic insight into gut physiological responses. Decreased transport of blackberry phenolics were observed with pretreatment, suggesting that chronic exposure led to decreased flux through the cell or possibly shifts in metabolism of phenolics. We also observed changes in expression of xenobiotic transport and metabolism genes with chronic exposure to blackberry extract that were consistent with the observed decreased transport of phenolic compounds. Although the transport data in this study did not appear to correlate to previous animal and clinical studies, this may be due to differences in phenolic class and matrix of the tested extract. Future studies should consider exploring methods to develop a model that incorporates other aspects of human physiology affected by chronic exposure to phenolics, such as the liver and the gut microbiome. Taken together, these results suggest that intestinal xenobiotic transport and metabolism are modulated by chronic exposure to phenolic-rich blackberry extract. Overall, these results indicate that adaptation occurring to the

intestinal epithelium with chronic exposure to phenolic compounds is critical to consider for a variety of implications involving absorption. As such, studies assessing bioavailability with only an acute dose may not be readily translational due to differences in consumers and non-consumers or diets rich in phenolic compounds.

CHAPTER 5. EFFECT OF 3-WEEK BLACKBERRY REPEATED EXPOSURE ON THE ABSORPTION, METABOLISM, AND EXCRETION OF FLAVONOIDS IN LEAN AND OBESE HUMANS

5.1 INTRODUCTION

Increased consumption of berry fruits has been associated with a reduced incidence of certain chronic diseases, such as heart disease and type 2 diabetes (Cassidy et al., 2013; Muraki et al., 2013). Supporting these associations are clinical studies linking improvements in markers of heart disease and oxidative stress to berry consumption (Del Bo' et al., 2015). Specifically, consumption of blueberry, cranberry and strawberry have all been reported to improve risk factors for cardiovascular disease such as high blood pressure, oxidized LDL, lipid peroxidation, and low HDL concentrations (Basu et al., 2010, 2014; Ruel et al., 2008). Though the mechanism of action involved in how the components in berries impart their observed beneficial effects is still a topic of much investigation, it is known that that berries are a rich dietary source of flavonoids, especially the pigmented anthocyanins, which have been hypothesized to be involved in exerting their biological activity.

Though not as heavily researched as the aforementioned berries, there is substantial *in vitro* work suggesting that blackberry can help prevent risk factors thought to be involved in cancer and heart disease, such as oxidized lipoproteins and nucleic

acids (Kaume et al., 2012). In pre-clinical rodent models, blackberry has been reported to have effects ranging from prevention of bone loss (Kaume et al., 2015) to decreased systemic blood glucose concentrations (Stefănuţ et al., 2013), while clinically it may improve risk factors for cardiovascular disease in dyslipidemic populations (Aghababaee et al., 2015). Since flavonoids appear to have strong potential as the mediators of the observed beneficial effects of berry consumption, it is critical to fully understand factors that influence or alter the absorption and excretion of these compounds, especially in the context of long-term dietary patterns that more closely resemble those linked to epidemiological associations.

While promising, application of berry-derived anthocyanins and other flavonoids for use in disease preventative strategies is limited by the notion that, excluding microbial metabolites, the acute oral bioavailability of intact flavonoids is less than 5% (Neilson and Ferruzzi, 2011). However, it is important to note that pharmacokinetic assessments commonly reported for flavonoids and used in of determining their bioavailability are typically performed using a single acute dose which is not representative of the chronic exposure resulting from broad dietary patterns. Indeed, both clinical and pre-clinical research has demonstrated that repeated exposure to flavonoids in a longer-term fashion resembling daily consumption dietary patterns may alter bioavailability of these compounds. For example, ten-day repeated administration of a grape seed extract product in rodents improved plasma response of catechin and epicatechin by greater than approximately 150% (Ferruzzi et al., 2009; Wang et al., 2012). Four-week daily administration of 800 mg of the flavan-3-ol epigallocatechin

gallate (EGCG) per day before an acute pharmacokinetic assessment resulted in plasma EGCG AUC values approximately 60% greater compared to those not repeatedly exposed to EGCG. Considering this potential for adaptation, a more complete understanding of factors driving this effect from whole foods, such as berries, is critical to best leverage this phenomenon for therapeutic endpoints.

In addition, due to the increasing prevalence of obesity on a global level (Ogden et al., 2007), it is important to consider how this condition may affect bioavailability of flavonoids and their specific metabolites. This information is critical to develop targeted dietary recommendations for these population groups. Certain factors associated with obesity have been implicated in possibly altering the bioavailability or bioactivity of these compounds (Xiao and Högger, 2014), including alteration of gut function and modulated gut bacterial populations that may affect production of various bioactive metabolites (Selma et al., 2015). Additionally, obesity has been associated with decreased status of both fat-soluble (vitamin K, β-carotene and tocopherol) and water-soluble micronutrients (folate), suggesting that increased BMI may affect absorption and/or metabolism of dietary compounds (Mahabir et al., 2008; Shea et al., 2010; Wise et al., 2009). In order to better understand adaptation to repeated berry exposure in lean and obese participants, the present study tested whether 3-week repeated daily exposure to blackberries elicits an adaptive response in the absorption and/or excretion of flavonoids as assayed by a seven-hour pharmacokinetic challenge. We hypothesized that there would be an adaptive response in the absorption and excretion of flavonoids

with repeated exposure to blackberry and that this response will differ between lean and obese groups volunteers.

5.2 Materials and Methods

5.2.1 Reagents

Analytical standards of cyanidin-3-*O*-glucoside (Chromadex, Irvine, CA), epicatechin, and kaempferol-7-*O*-glucoside (Sigma, St. Louis, MO) were obtained. Solvents for LC-MS/MS were of mass spectrometry grade and all solvents were at least analytical grade. Formic acid and triflouroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO).

5.2.2 Experimental study design and diets

This randomized, controlled, cross-over design study was conducted at the USDA Beltsville Human Nutrition Research Center (BHNRC; registered at ClinicalTrials.gov#: NCT01944579). This study (see **Figure 19** for study design) received IRB approval (see Appendix C), and participants provided written informed consent. Inclusion criteria included those 25-75 years old. Exclusion criteria included: individuals using blood-thinning medications, presence of any gastrointestinal disease, metabolic disease, or malabsorption syndromes, pregnancy during the previous 12 months, are currently pregnant or lactating, or plan to become pregnant during the study, Type 2 diabetes requiring the use of oral antidiabetic agents or insulin, fasting triglycerides greater than

300 mg/dL, fasting glucose greater than 126 mg/d, use of prescription or over-the-counter antiobesity medications or supplements during and for at least 6 months prior to the start of the study or a history of a surgical intervention for obesity, active cardiovascular disease, use of any tobacco products in past 3 months, unwillingness to abstain from herbal supplements for two weeks prior to the study and during the study, and known (self-reported) allergy or adverse reaction to blackberries or other study foods. Participants were provided a controlled diet generated by a registered dietitian during the length of the study to maintain weight and to provide dietary flavonoids from blackberry (See **Table 14** for detailed meal composition and **Figure 18** for macronutrient composition). Each diet provided approximately 15% energy as protein, 30% energy as fat, and 55% energy as carbohydrate. Breakfast and dinner meals were required to be consumed under staff supervision at the research center on weekdays. Blackberries were a combination of *Rubus laciniatus*, *Rubus* Marion, and *Rubus fruticosus* L. purchased from SYSCO (Houston, TX). A total of 18 participants including individuals with normal BMI (n = 7, BMI 20-25) and an obese BMI (n = 11, BMI > 30) were fed a controlled diet that included either 300 g/d blackberries total, split between breakfast and dinner meals (treatment) or an isocaloric amount of gelatin (272 g) as the control. Treatment order was randomized, with half of the participants receiving the control treatment first over three weeks and the other half blackberries first over three weeks. A three-week washout period where the participants were instructed to avoid berries was between treatments. Each participant received the same lot of berries during the testing day, as described in the pharmacokinetic assessment section below.

Table 14. Example meal composition of a typical day's diet provided to participants.[a]

Date:	Blackberry Microbiota Study- SUNDAY										
	CONTROL										
	TREATMENT										
INITIALS	Production Numbers (TOTAL)										
		L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
	Breakfast										
	Waffles- AUNT JEMIMA	60	67	73	80	86	94	100	107	113	120
	Promise Buttery Spread	6	7	7	8	9	9	10	11	11	12
	Syrup, pancake	18	20	22	24	26	28	30	32	34	36
	Sausage, turkey patty- Jimmy Dean	60	67	73	80	86	94	100	107	113	120
	Milk, 2%	128	142	156	170	184	200	214	228	242	256
	BLACKBERRY, FROZEN, UNSWEETENED	150	150	150	150	150	150	150	150	150	150
	STRAWBERRY JELLO	136	136	136	136	136	136	136	136	136	136
	Lunch										
	Kaiser Roll- Ottenbergs	56	62	68	74	81	87	94	100	106	112
	Beef BBQ- Brookwood Farms	80	89	98	106	115	125	134	142	151	160
	Avocado Study- Potato Salad	99	110	121	132	143	154	165	176	187	198
	Peaches, DOLE	80	89	98	106	115	125	134	142	151	160
	Dinner										
	Spaghetti- BARILLA	90	100	110	120	130	140	150	160	170	180
	Spaghetti Sauce w/meat- RAGU	70	78	85	93	101	109	117	125	132	140
	Mozzarella Cheese, Part-Skim, Shredded	18	20	22	24	26	28	30	32	34	36
	Vegetables, mixed, fzn	80	89	98	106	115	125	134	142	151	160
	Cool Whip, lite	10	11	12	13	14	16	17	18	19	20
	BLACKBERRY, FROZEN, UNSWEETENED	150	150	150	150	150	150	150	150	150	150
	STRAWBERRY JELLO	136	136	136	136	136	136	136	136	136	136
	Evening Snack				0						
	Celery, stalk	55	61	67	73	79	86	92	98	104	110
	Salad Dressing, ranch	32	36	39	43	46	50	53	57	60	64
	Cracker, cheese, gold fish	28	31	34	37	40	44	47	50	53	56

a) "L" indicates the various "levels" of energy amounts provided to participants to maintain body weight.

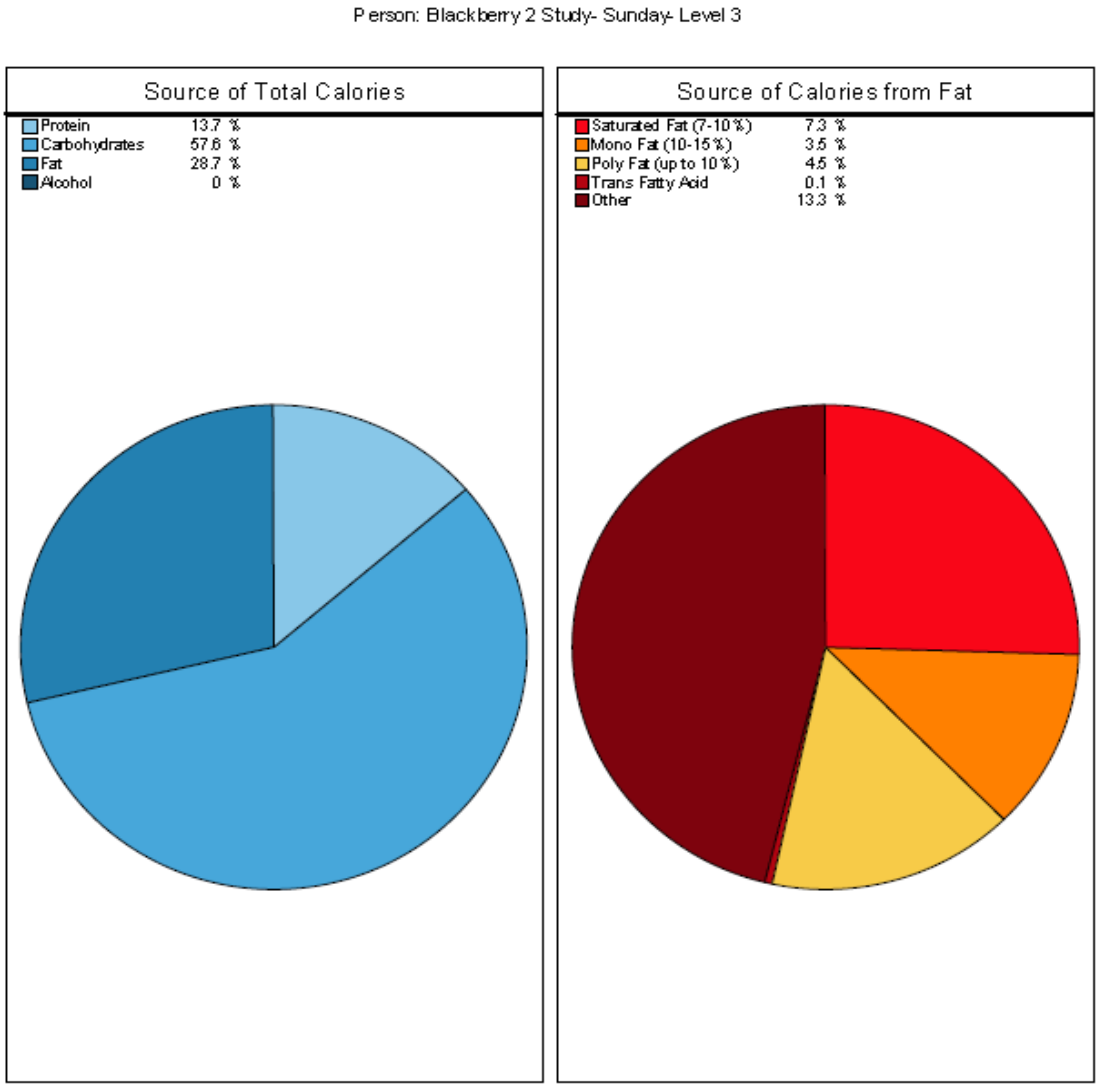


Figure 18. Macronutrient distribution of a typical day's diet.

5.2.3 Pharmacokinetic (PK) assessment

In order to determine the effect of repeated blackberry exposure on flavonoid bioavailability and metabolism, a pharmacokinetic (PK) assessment was conducted both three weeks after the participants received the control diet and three weeks after consuming the diet supplemented with blackberry. During the day of the PK assessment, volunteers arrived at the beginning of the day at the facilities fasted, produced a baseline urine, and then consumed 300 g blackberry homogenate within 5 min. Low-flavonoid meals were provided at lunch at dinner times. Blood draws were performed at 0, 0.33, 0.66, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7 h and urine was collected at 0, 1.5, 3, 4.5, 6, and 7 h. After blood was centrifuged at 2560 *g* for 10 min, 1 mL aliquot of plasma collected and stabilized with 0.6mL of 0.44 M triflouroacetic acid for anthocyanin analysis, and another 1 mL plasma aliquot was stabilized with 1% ascorbic acid for flavan-3-ols and flavonol analysis. Biological samples were stored at -80 °C until analysis.

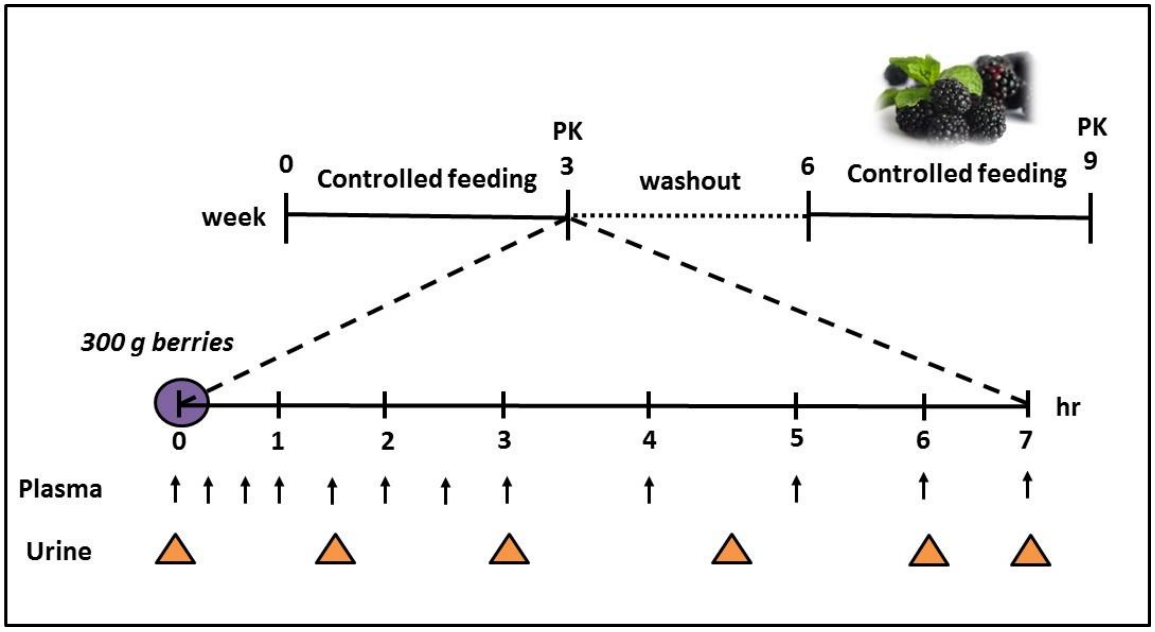


Figure 19. Experimental study design for blackberry clinical trial. Lean (BMI 20-25; n = 7) and obese (BMI > 30; n = 11) participants were recruited for a randomized, cross-over, controlled feeding study to assess flavonoid pharmacokinetics after a three-week control period and after a three-week period with 150 g blackberries provided at both breakfast and dinner meals. A washout period where the participants were instructed to avoid berries was included between treatments. Pharmacokinetics were assessed from 0-7 h after being placed on a controlled feeding regiment for three weeks containing either 277 g berry-flavored gelatin (control) or 300 g blackberries daily (containing an average of 753 mg flavonoids). Plasma and urine were collected during the pharmacokinetic challenge and analyzed by LC-MS/MS.

5.2.4 Solid phase extraction (SPE)

5.2.4.1 Anthocyanin SPE

Solid phase extraction (SPE) for flavonoids was performed as previously reported with some modification (Blount et al., 2015). For plasma anthocyanin analysis, 1 mL plasma was loaded onto a 30 mg Oasis HLB cartridge (Waters Co., Milford, MA) that was activated with 3 mL MeOH followed by 3 mL H_2O. The cartridge was then washed with 2

mL of 2% formic acid:H_2O (v:v), then 1 mL 95:5 H_2O:MeOH (v:v), and was finally eluted with 2 mL 2% formic acid:MeOH (v:v). 10 µL of 10 µM ethyl gallate was added to the eluate as an internal standard for MS, and this was dried under vacuum (< 50 mm Hg) at 37°C. Residuals were then resolubilized in 100 µL of 2% formic acid:H_2O (v:v), sonicated and vortex-mixed for 10 s each, and then centrifuged at 18,000 *g* for 5 min before supernatant was transferred to a 300 µL HPLC vial for analysis. Extraction recovery was assessed using ethyl gallate and was found to be greater than 95%.

For urine analysis, 10 mL urine was centrifuged for 2 min at 2,000 *g* to remove particulates and was then loaded onto a 150 mg Oasis HLB cartridge (Waters, Milford, MA) that was activated with 6 mL MeOH followed by 6 mL H_2O. This was followed by the cartridge being washed with 4 mL of 2% formic acid:H_2O (v:v), 2 mL 95:5 H_2O:MeOH (v:v), and then eluted with 4 mL 2% formic acid:meOH (v:v). 50 µL of 10 µM ethyl gallate was then added to eluate as an internal standard for MS and was dried under vacuum. Residuals were then resolubilized as above and centrifuged at 18,000 *g* for 10 min.

5.2.4.2 Flavan-3-ols and Flavonols SPE

SPE of plasma containing flavan-3-ols and flavonols was the similar as above with some modification. After plasma was loaded onto a cartridge activated with 1 mL MeOH followed by 1 mL H_2O, the cartridge was washed with 1 mL of 1.5 M formic acid:H_2O (v:v), 1 mL 95:5 H_2O:MeOH (v:v), and then was eluted with 2 mL 0.1% formic acid:MeOH (v:v). Ethyl gallate internal standard was added to the eluate and dried under vacuum.

Residuals were then resolubilized in 160 µL of 0.1% formic acid:H_2O (v:v) + 40 µL of 0.1% formic acid:ACN (v:v) before continuing procedure as in the section above.

For urine analysis, SPE procedure was identical as the section above except for a few modifications. 10 mL urine was loaded onto a 150 mg Oasis HLB cartridge that was activated with 5 mL MeOH followed by 5 mL H_2O. The cartridge was then washed with 2 mL of 1.5 M formic acid, 5 mL 95:5 H_2O:MeOH (v:v), and then was eluted with 4 mL 2% formic acid:MeOH (v:v). Ethyl gallate internal standard was added to the eluate and then dried under vacuum.

5.2.5 LC-MS/MS analysis

Biological samples were initially screened for a various parent compounds and their methyl, sulfate, and glucuronide metabolites, after which the major metabolites were followed.

5.2.5.1 Anthocyanin analysis

Parent compounds and metabolites were separated using an Agilent 1200 HPLC system using a Waters XBridge BEH shield RP-C18 XP 2.1 x 100 mm 2.5µm column following a 2 µm frit filter. The column and autosampler were maintained at 40 °C and 10 °C, respectively. The flow was set to 0.26 mL/min and mobile phases were A: 2% formic acid (aq.); B: 0.1% formic acid/acetonitrile. Gradient conditions were 5% B at 0 min, 25% B at 15 min, 30% B at 18 min, 5% B at 19 min, and 5% B at 23.5 min to reset the gradient.

5.2.5.2 Flavan-3-ol and flavonol analysis

Flavan-3-ols and flavonols were analyzed similarly as anthocyanins except for a few differences. Compounds were analyzed in negative mode and different chromatography conditions are as follows. The flow was set to 0.26 mL/min and mobile phases were A: 0.1% formic acid (aq.); B: 0.1% formic acid/acetonitrile. Gradient conditions were 5% B at 0 min, 60% B at 10 min, 95% B at 11 min, 5% B at 12 min, and hold at 5% B at 22 min to reset the gradient.

5.2.6 LC-MS/MS for flavonoid quantification

Analytes were quantified using liquid chromatography with tandem mass spectrometry (LS-MS/MS) using an Agilent 6460 triple quadrupole with electrospray ionization (ESI) operated in negative mode using selected reaction monitoring (SRM) mass transitions. Injection volume was 10 µL for plasma and 5 µL for urine samples.

Acquisition SRMs for all analytes are listed in **Table 15**. In addition, both methods had an SRM for ethyl gallate in negative mode, 197 → 169 *m/z* for use as an internal standard for MS. All mass transitions used a dwell time of 200 ms and fragmentor voltage of 135 V, and collision energy of 17 eV. Source parameters were as follows: gas temperature = 350 °C, gas flow = 11 L/min, nebulizer pressure = 30 psi, sheath gas temperature = 350 °C, sheath gas flow = 11 L/min, capillary potential = 3500 V, and nozzle voltage = 1000 V.

5.2.7 Limit of detection and quantification

Limit of detection (LOD) was defined as signal-to-noise ratio (S/N) = 3 and limit of quantification (LOQ) as S/N = 9. LOD for cyanidin-3-*O*-glc, epicatechin, kaempferol-7-*O*-glucoside, and quercetin-3-*O*-glucoside were 0.24 nM, 22.9 nM, 5.1 nM, and 1.5 nM, respectively. LOQ was 0.72 nM, 68.7 nM, 15.3 nM, and 4.5 nM, respectively. Both values were obtained by producing serial dilutions of the analyte. Signal-to-noise (S/N) ratio was calculated using MassHunter software using the peak-to-peak method.

Table 15. Acquisition SRMs for LC-MS/MS.

Compound	MS1	MS2	mode
Peonidin-glucur	477	301	+
Cyanidin-glucur	463	287	+
Peonidin-glc	463	301	+
Cyanidin-3-*O*-glc	449	287	+
EC-3'-*O*-glucr	465	289	-
Kaemp-glucr	461	285	-
Kaemp-7-*O*-glc	447	285	-
Methyl-Kaemp-glucr	475	299	-

5.2.8 Data analysis

Plasma AUC is expressed as nmol compound·h and was normalized to body weight and blood volume. Normal physiological parameters (blood volume/body weight) vary for obese patients, so an adjusted calculation was used in this case (Lemmens et al., 2006). Urinary accumulation is expressed at cumulative nmol compound. Since different lots of blackberries were used throughout the study, data were also normalized on the basis of flavonoid dose. In addition, a carryover subtraction was performed as to eliminate bias due to accumulation of flavonoids throughout the blackberry treatment period. Any non-normally distributed data (as determined by the goodness-of-fit test) was transformed using the optimum lambda Box-Cox value, after which normality was again tested. Statistical significance was then determined using a paired t-test. Analysis was performed using JMP 12 (SAS Institute, Cary, NC), and significance was set at $\alpha = 0.05$.

5.3 Results

5.3.1 Key flavonoid composition of blackberries

Phenolic composition of blackberries is shown in **Table 16**. The main anthocyanin in the blackberries was cyanidin-3-*O*-glucoside, which is consistent with previous reports (Kaume et al., 2012; Mertz et al., 2007). Interestingly, the blackberries used in this study contained greater amounts of kaemperfol glycosides and less quercetin glycosides compared to previous reports (Mertz et al., 2007), but this variation may be related to differences in the specific varieties and harvest season.

5.3.2 Characterization of parent flavonoids and metabolites

Figure 20 displays representative chromatograms of anthocyanins and their metabolites. In addition to the unmetabolized cyanidin-3-*O*-glc being identified, methyl and glucuronide derivatives were also detected. Since native peonidin was not detected to any great extent in blackberry, the peonidin in circulation is most likely derived from methylation of cyanidin by Phase II metabolizing systems, such as catechol-*O*-methyltransferase (COMT). In addition, the presence of the two unresolved peaks likely indicates methylation at two different sites on the anthocyanin structural backbone.

Figure 21 shows representative chromatograms of flavan-3-ols, flavonols and their metabolites. As with the anthocyanins, both methyl and glucuronide derivatives of kaempferol were detected. Presence of epicatechin-3'-*O*-glucuronide in urine and plasma was confirmed by comparing retention times to an authentic standard synthesized using the human UGT1A9 enzyme (Blount et al., 2012) (data not shown).

Table 16. Composition of key blackberry flavonoids from an example batch.[a]

	Cy-3-*O*-glc	Catechin	Epicatechin	Kaemp-7-O-glc	Quercetin-gly 1	Quercetin-gly 2
Batch	**ug/g fresh weight**					
A	932 ± 48.4	111 ± 7.22	638 ± 43.1	1440 ± 68.3	112 ± 4.66	45.0 ± 2.01
B	585 ± 14.1	135 ± 2.09	637 ± 22.3	910 ± 22.6	54.9 ± 2.18	38.1 ± 1.41
C	555 ± 45.4	157 ± 9.25	739 ± 59.2	919 ± 63.2	75.2 ± 5.15	44.9 ± 3.19
D	322 ± 10.9	45.9 ± 2.60	237 ± 13.5	477 ± 28.7	108 ± 6.13	51.2 ± 2.52
E	617 ± 2.16	99.1 ± 3.98	1100 ± 14.1	982 ± 6.30	87.1 ± 0.362	45.3 ± 7.37
AVG	602	219	670	886	87.4	44.9

[a]Values are expressed average ± SEM (n = 3).

5.3.3 Plasma flavonoid AUC

Figure 22 displays the absorption, metabolism, and excretion of select anthocyanins detected in plasma over 7 h after a bolus dose of blackberries. Overall, repeated blackberry treatment appeared alter the absorption and metabolism of select anthocyanins as indicated by differences in plasma AUC. In comparing specific treatments, the results showed that there was a statistically significant ($P < 0.05$) increase in plasma AUC of peonidin-glc 1 after 3-week blackberry treatment in lean but not obese participants. Pooling results from peonidin-glc 1 plus 2 plasma AUC, there was a borderline ($P = 0.056$) significant increase in these compounds for lean but not obese participants after blackberry treatment. In comparing differences in flavonoid plasma AUC due to BMI, a trend ($P = 0.099$) was observed for greater amounts of plasma

peonidin-glc 1 in the lean but not obese group after being exposed to blackberry. There was a stronger trend for the lean group containing higher amounts of cyanidin-3-*O*-glc in plasma than the obese population under control conditions ($P = 0.076$) compared to after blackberry exposure ($P = 0.11$). When results from total anthocyanin glucosides are pooled (cyanidin-3-*O*-glc plus methylated compounds), there was a subtle trend for higher circulating amounts of these compounds in the lean compared to the obese group under both control ($P = 0.10$) and blackberry ($P = 0.11$) conditions.

Figure 23 shows the absorption, metabolism, and excretion of flavan-3-ols and flavonols over 7 h after a bolus dose of blackberries under both control and treatment conditions. Results mainly showed a trend for higher plasma AUC of pooled kaempferol and its derivatives (parent compounds and metabolites) in the lean compared to the obese group after blackberry exposure ($P = 0.082$), but this trend was less strong for control ($P = 0.19$).

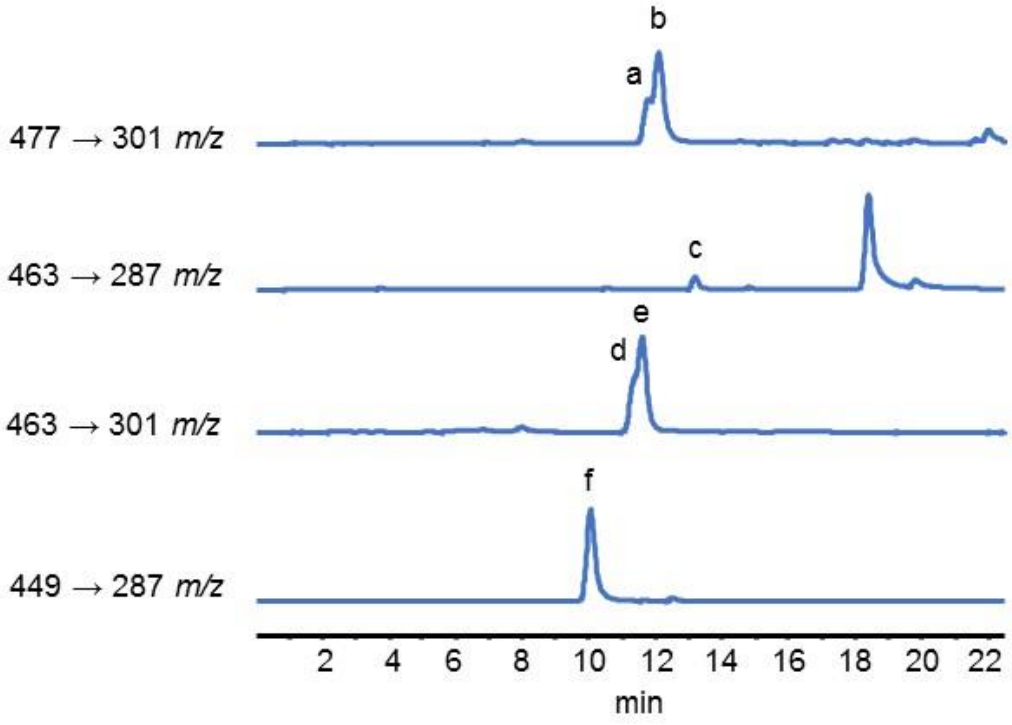


Figure 20. Representative chromatograms of anthocyanins and their metabolites identified in plasma and urine in positive mode. Peak identification: (a) peonidin-glucr 1; (b) peonidin-glucr 2; (c) cyanidin-glucr; (d) peonidin-glc 1 (e) peonidin-glc 2; (f) cyanidin-3-glc.

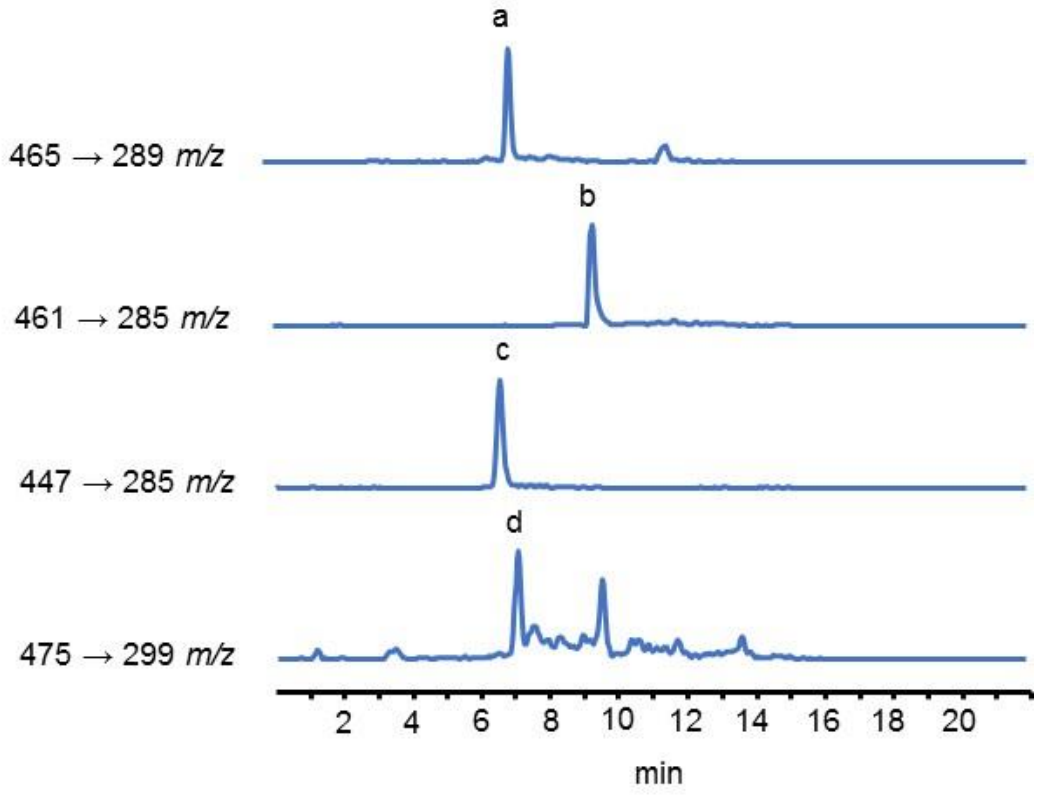


Figure 21. Representative chromatograms of flavan-3-ols, flavonols and their metabolites identified in plasma and urine in negative mode. Peak identification: (a) Epicatechin-3'-*O*-glucr; (b) Kaempferol-*O*-glucr; (c) Kaempferol-7-*O*-glc; (d) methyl-Kaempferol-*O*-glucr.

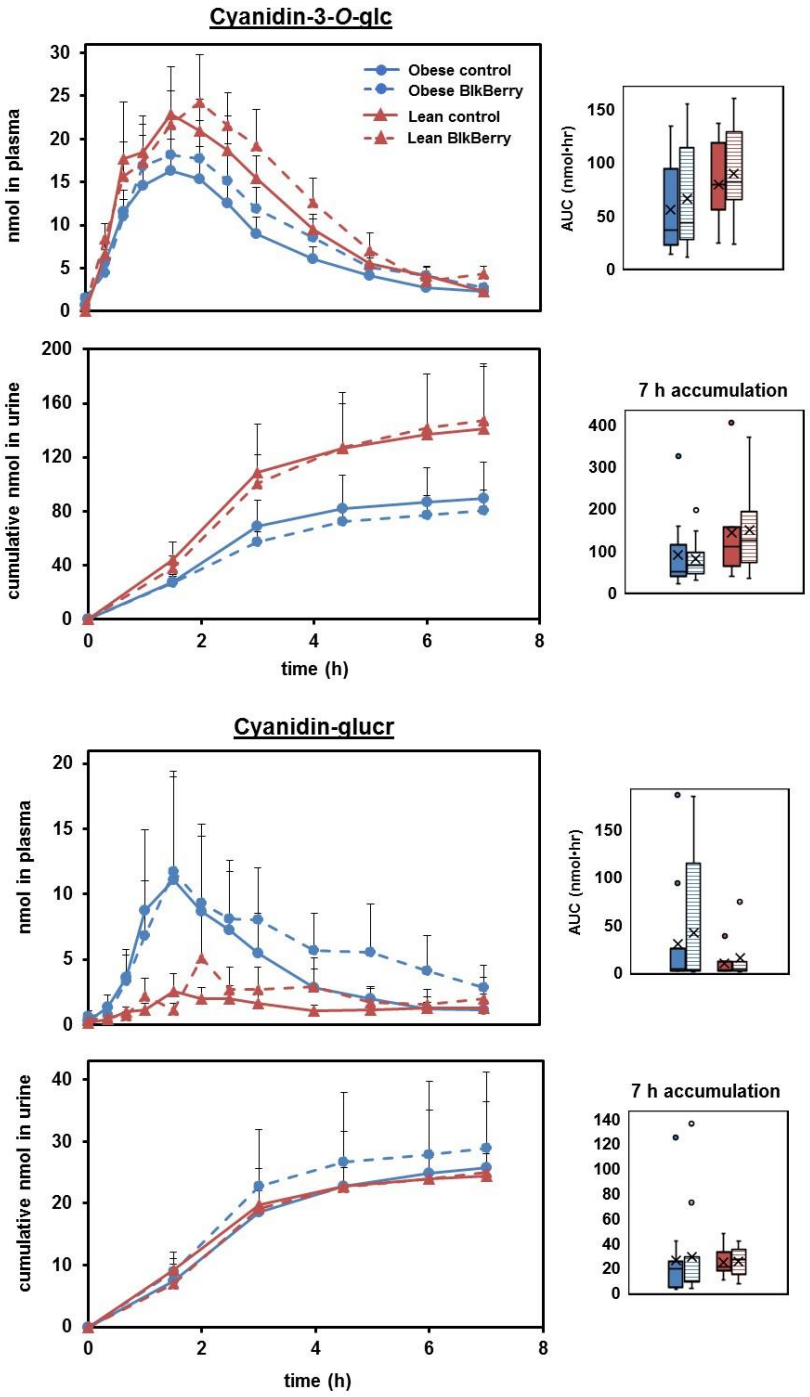
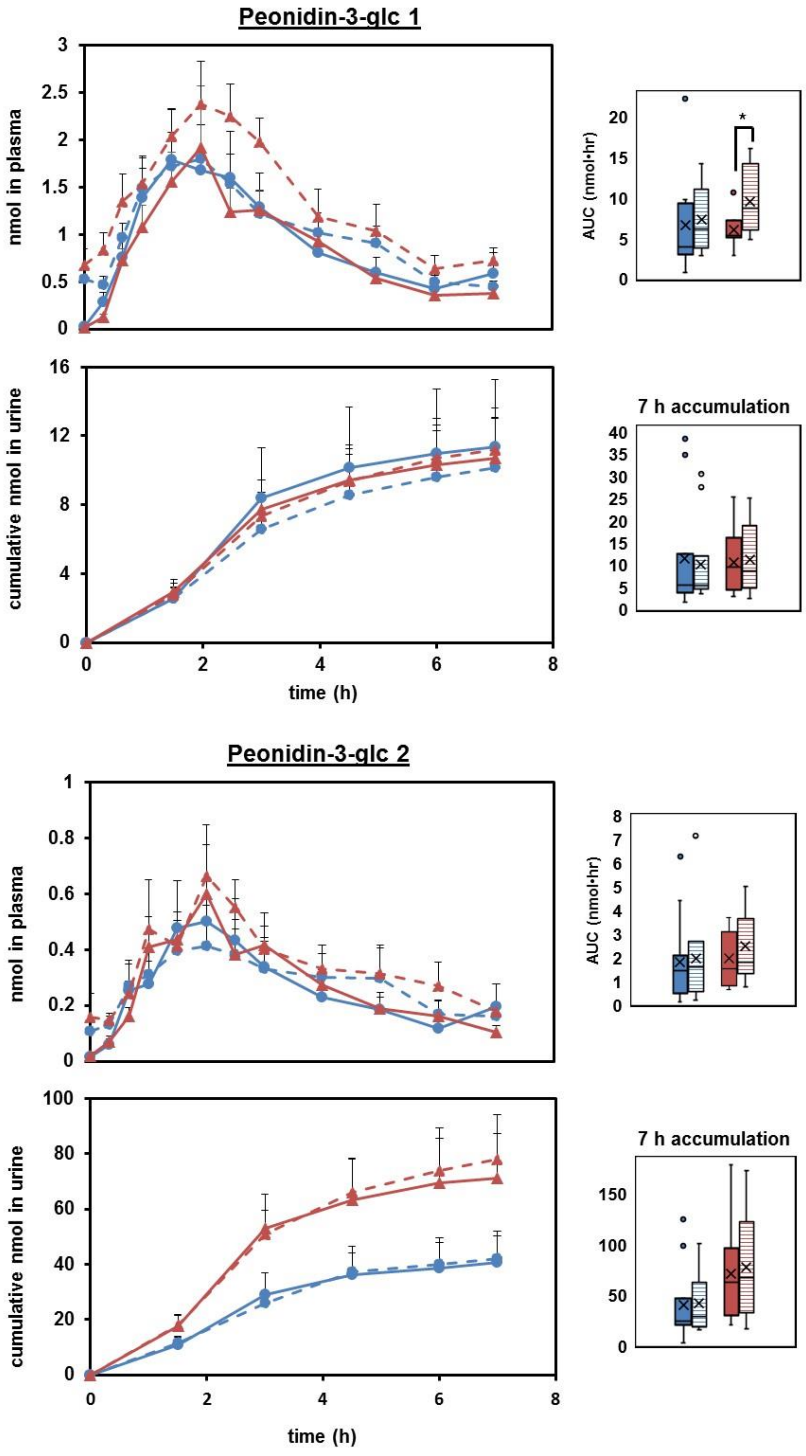


Figure 22. Absorption, metabolism, and excretion of anthocyanins after a bolus dose of blackberries. An acute pharmacokinetic assessment determined anthocyanin appearance in plasma and accumulation in urine over 7 h in both lean (n = 7) and obese (n = 11) populations. Data are displayed as average ± SEM. Box plots display interquartile range, median, and average as represented by an "x." *$P < 0.05$.



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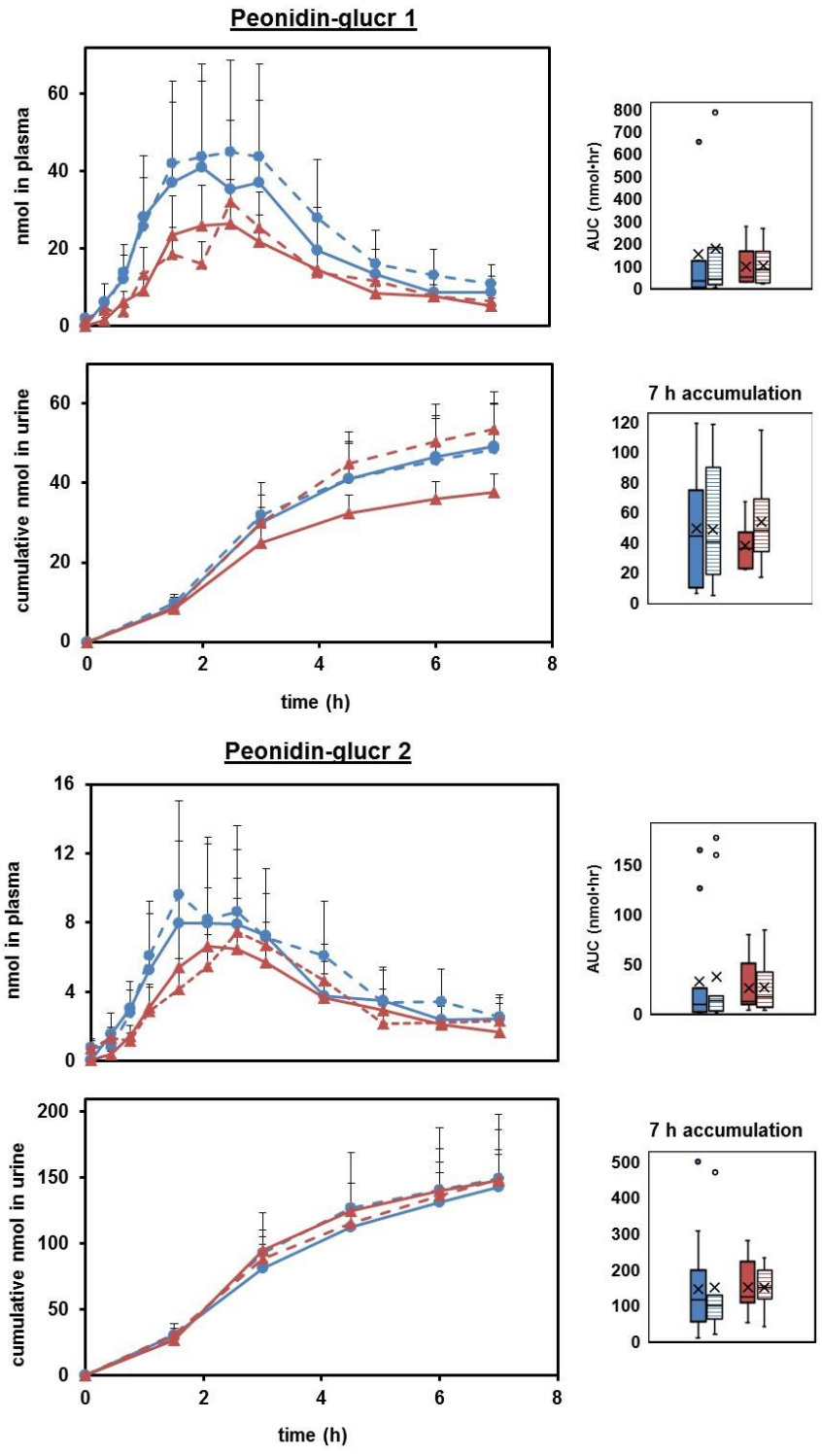

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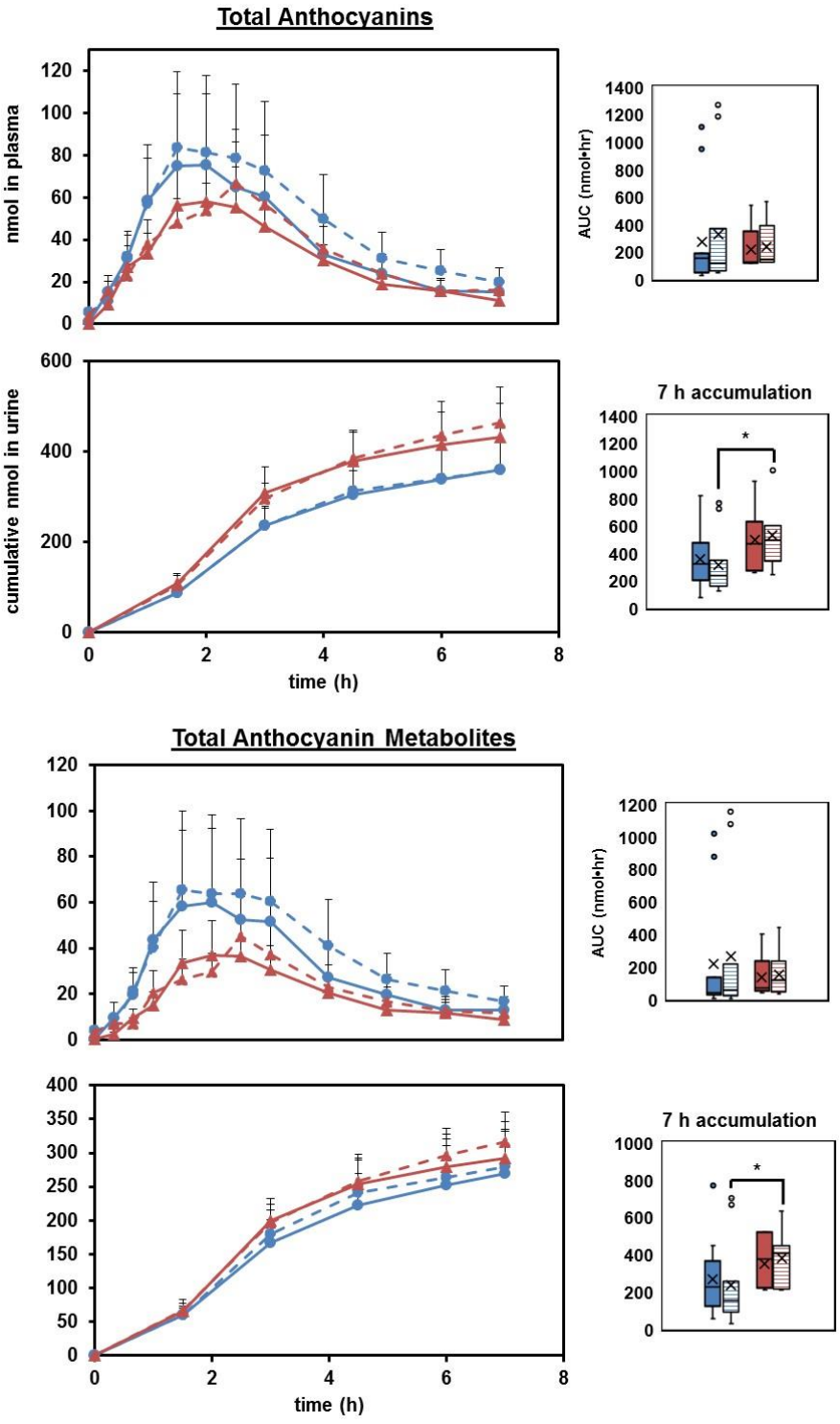

Figure 22, con'd.

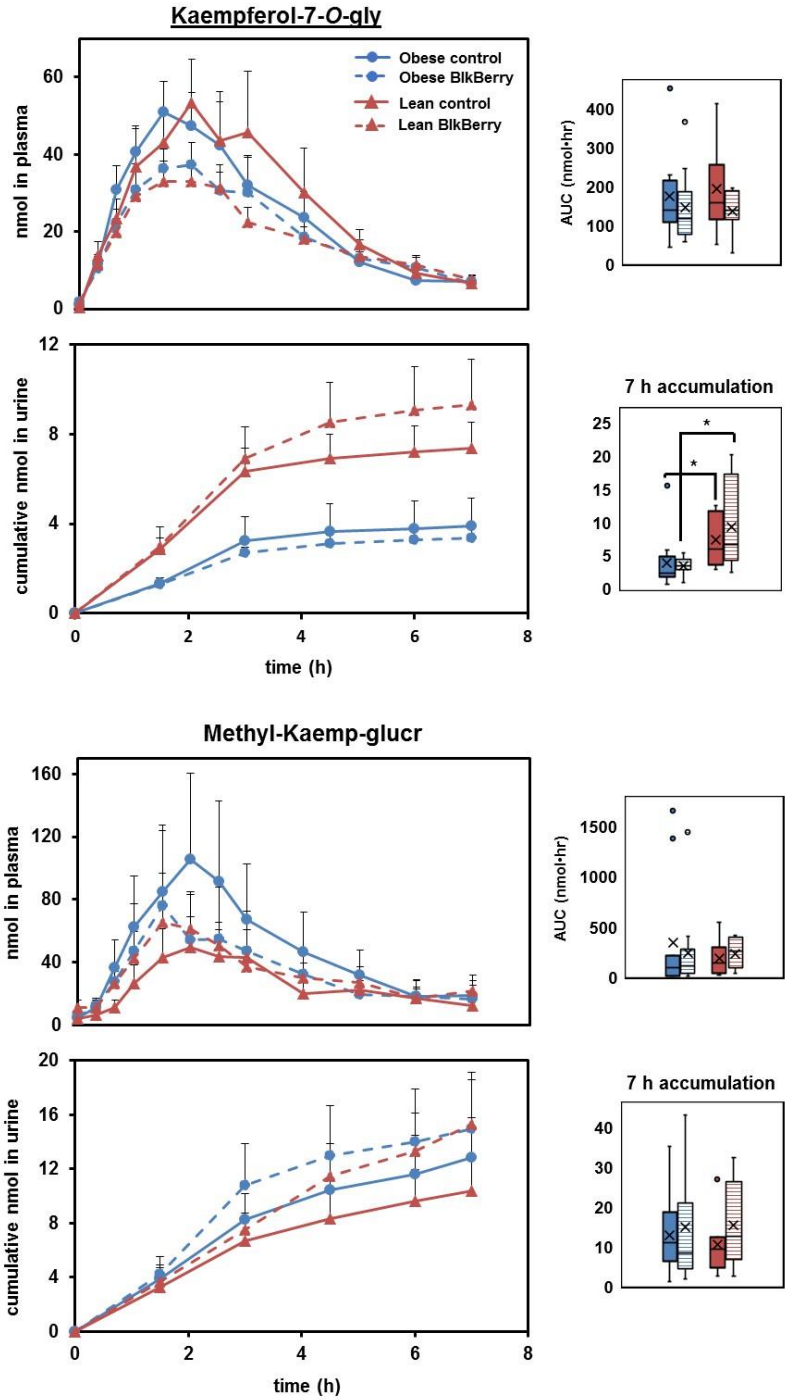

Figure 23. Absorption, metabolism, and excretion of flavan-3-ols and flavonols after a bolus dose of blackberries. An acute pharmacokinetic assessment determined flavan-3-ol and flavonol appearance in plasma and accumulation in urine over 7 h in both lean (n = 7) and obese (n = 11) populations. Data are displayed as average ± SEM. Box plots display interquartile range, median, and average as represented by an "x." *P < 0.05.

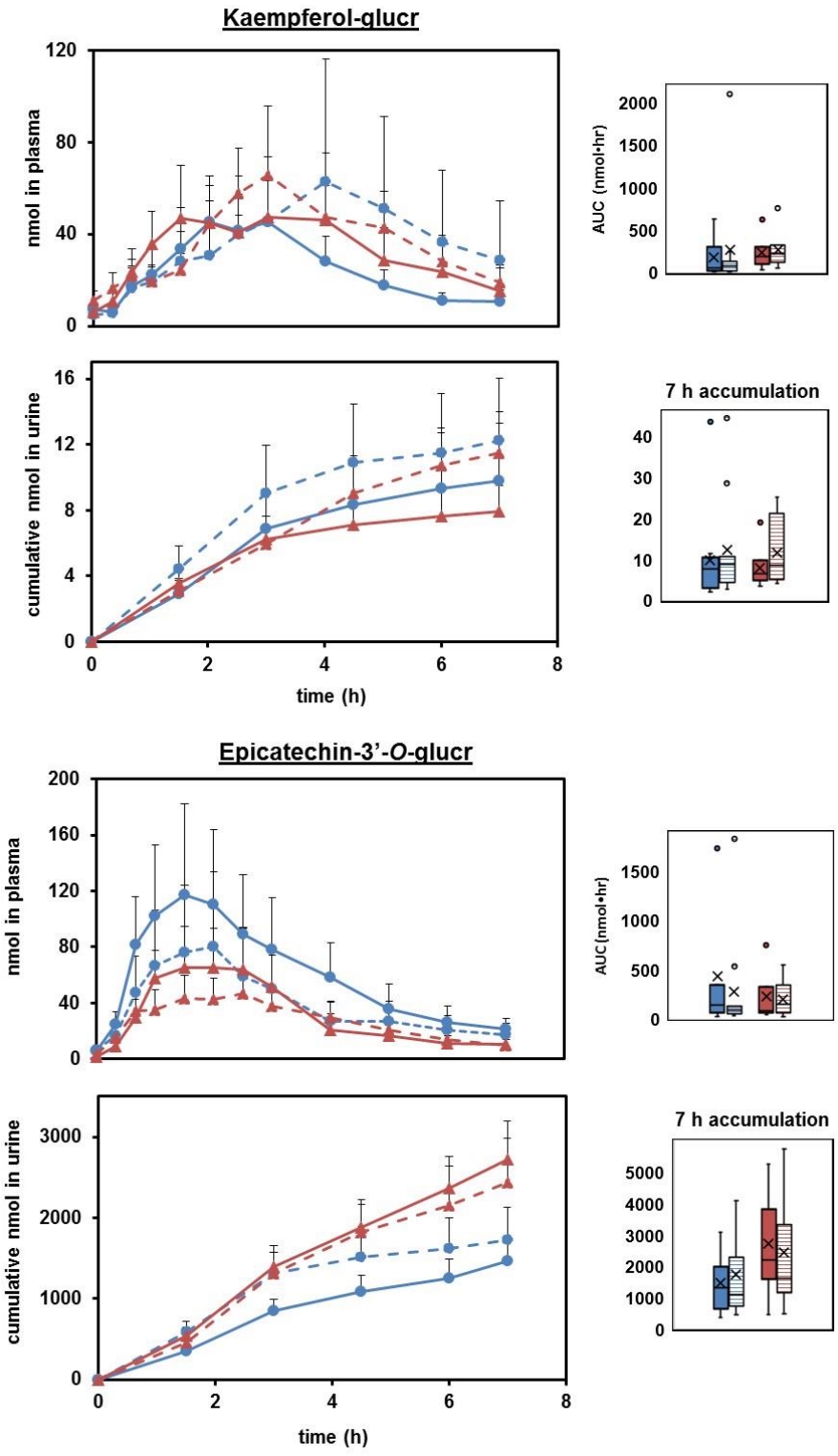

Figure 23, con'd.

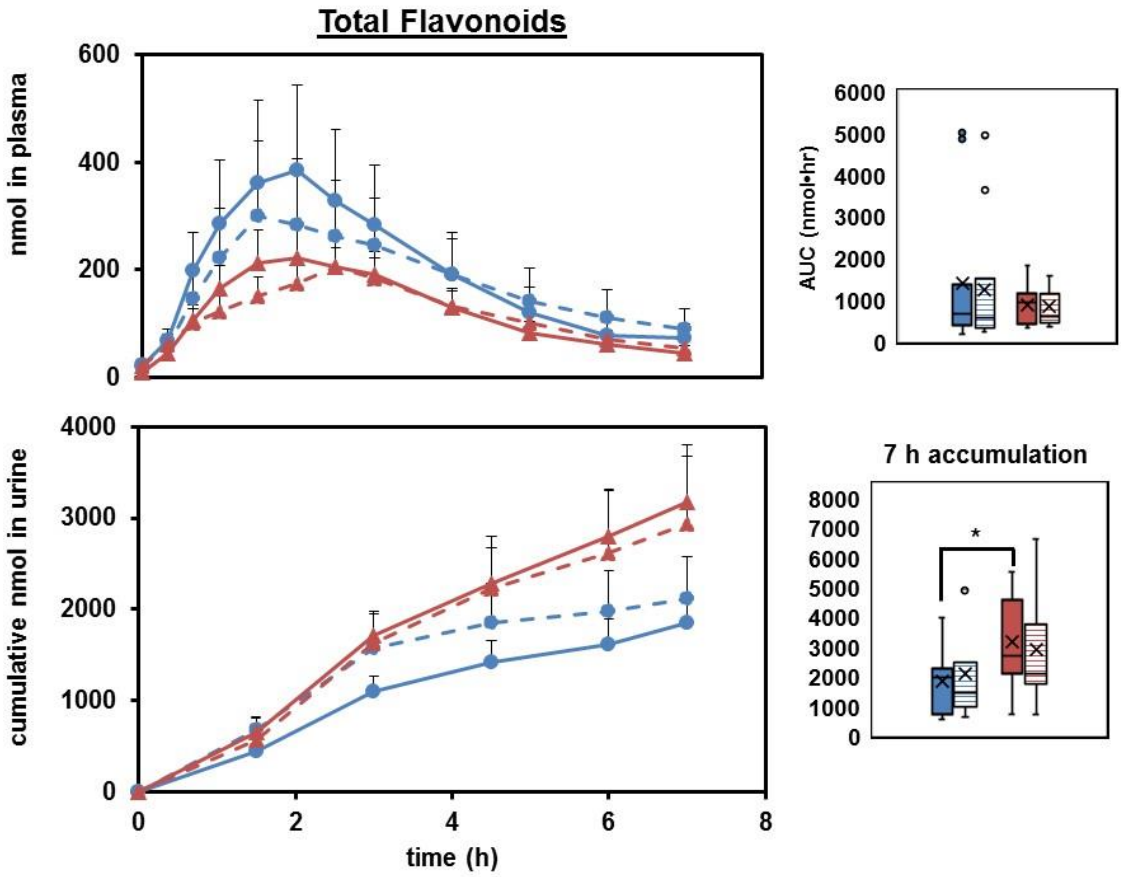

Figure 24. Absorption, metabolism, and excretion of total flavonoids (all analytes) after a bolus dose of blackberries. An acute pharmacokinetic assessment determined flavonoid appearance in plasma and accumulation in urine over 7 h in both lean (n = 7) and obese (n = 11) populations. Data are displayed as average ± SEM. Box plots display interquartile range, median, and average as represented by an "x." *$P < 0.05$.

5.3.4 Flavonoid accumulation in urine

Supporting results observed in plasma, there were differences in absorption and metabolism of select anthocyanins with BMI as indicated by clearance and accumulation of these compounds in urine (see Figures 22, 23 and 24). For individual anthocyanins, there was a trend for the lean group to have greater urinary accumulation of cyanidin-3-*O*-glc than obese volunteers under both control ($P = 0.079$) and blackberry treatment ($P = 0.068$) conditions. In addition, there was a trend for greater amounts of urinary peonidin-glc 2 in the lean compared to the obese group under both control ($P = 0.056$) and blackberry treatments ($P = 0.059$). There was significantly greater total anthocyanin metabolite urinary accumulation in lean compared to obese volunteers after blackberry treatment ($P = 0.015$) but not under control conditions ($P = 0.074$). Finally, when results for all anthocyanins were pooled, there was significantly greater urinary accumulation in lean compared to obese participants with blackberry exposure ($P = 0.016$) but not under control conditions ($P = 0.081$).

For kaempferol-7-*O*-glycoside, there was significantly greater urinary accumulation in lean compared to the obese volunteers under both control ($P = 0.013$) and blackberry ($P = 0.011$) conditions. No differences in epicatechin metabolism was observed with blackberry treatment or BMI. Interestingly, when data from all analytes (total flavonoids) are pooled, there was significantly greater urinary accumulation in lean compared to obese participants under control ($P = 0.037$) but not blackberry conditions ($P = 0.17$). There were no statistically significant differences when comparing differences in urinary accumulation before and after blackberry treatment within groups.

5.4 Discussion

Though the experimental paradigm of an acute PK assessment is most commonly used to measure the bioavailability of flavonoids and other bioactive compounds, data resulting from these acute experiments may not be representative of chronic dietary exposures to flavonoids occurring in broader populations. For instance, a clinical study with a two week run-in period providing 800 mg EGCG per day resulted in an approximately 60% increase in plasma AUC compared to those not exposed to EGCG (Chow et al., 2003). An additional clinical trial detected increases in circulating and excreted flavan-3-ols after 7-day repeated exposure to tea (Fung et al., 2013), though these results were not corrected for accumulation as in the present study. Our results showed differences in the absorption and metabolism of select anthocyanins with a 57% increase in the average plasma AUC of a methylated cyandin derivative (peonidin-glc 1) in lean participants after blackberry treatment, in addition to a trend for increased amounts of total methylated cyanidin derivatives (peonidin-glc 1 plus 2) in the plasma of this same group.

In contrast, Jean et al. (2012) study found no difference in cyanidin-3-*O*-gluoside plasma AUC after participants were given 1 g of black bean seed coat extract daily for two weeks (Jeon et al., 2012). However, Jeon et al. only measured cyanidin-3-*O*-glucoside and therefore these results may not have captured likely shifts in the metabolism of this anthocyanin after the period of repeated exposure. In addition, our study did not utilize isolated anthocyanins, but rather a whole food, which generally results in lower absorption, and potentially reduced metabolism, compared to isolated

flavonoids (Clifford et al., 2013). These differences in experimental design may also indicate that adaptation in absorption and metabolism may differ depending on whether a flavonoid is provided in a purified/extract form or in the context of a diet which may result in interactions between food macro- and micronutrients (Bordenave et al., 2014; Guo et al., 2013; Peters et al., 2010), in addition to synergistic properties of flavonoid mixtures (Tagashira et al., 2012). Ultimately, it is crucial to consider absorption and metabolism of these compounds in the context of a whole food, where there can be multiple interactions between food components and flavonoids (Zhang et al., 2004; Zhu et al., 2008).

Our data appear to demonstrate differential adaptation in the metabolism of anthocyanins with repeated exposure to blackberry between lean and obese subjects. For example, there was significantly greater total anthocyanin metabolite urinary accumulation in lean compared to obese volunteers after blackberry treatment, while there was no difference under control conditions. In contrast, there was no statistically significant difference in urinary accumulation of unmetabolized cyanidin-3-*O*-glc between groups after blackberry treatment or during the control period. Thus, the increase in the accumulation of anthocyanin metabolites in lean but not obese participants after blackberry exposure may indicate Phase II systems being differentially induced with repeated exposure due to BMI. Though there may be differences in urinary output, the values presented are volume corrected. The lack of a similar shift in the anthocyanin metabolites of the obese group may support the notion of a fundamental

difference in the metabolism of these compounds in various disease states such as obesity and diabetes (Xiao and Högger, 2014).

The metabolites detected in this study align with previous reports that observed key Phase II metabolites of anthocyanins as the methyl and glucuronide derivatives, especially those found in urine (Felgines et al., 2005). In addition, a tracer study that provided ^{13}C-labeled cyanidin-3-*O*-glucoside to humans volunteers reported similar Phase II metabolites as identified in the present study, namely the glucuronide and methyl glucuronide metabolites (Czank et al., 2013; de Ferrars et al., 2014). Finally, a study that provided an acute dose of 200 g of blackberries to humans observed glucuronide and methyl glucuronides as the major Phase II metabolites (Felgines et al., 2005), which corresponds with our analysis.

In regards to the metabolite profile, it appears that there is greater inter-individual variability in the metabolite concentrations with repeated exposure to blackberry, especially in regards to the anthocyanins (see box plots in Figure 22, 23, 24). This variability in metabolites appears to indicate differences in the adaptive response, which may reflect genetic polymorphisms in the xenobiotic metabolizing systems that result in a system more or less susceptible to induction (Ramos et al., 2014).

Experiments reported in Chapters 4 and 5 of this dissertation modeled the repeated exposure paradigm in humans intestinal Caco-2 cells to determine whether adaptation in flavonoid transport and metabolism occurred with repeated exposure. Chapter 4 demonstrated that pretreatment with isolated flavan-3-ols increased appearance of methylated derivatives, which corresponds to the increases in peonidin

levels observed in plasma with repeated exposure. Additionally, there were increases in transport of select flavan-3-ols when cell monolayers were pretreated with tea and grape seed extracts. In Chapter 5, experiments utilized a composite blackberry extract produced from the berries used in this clinical study. Interestingly, pretreatment with the extract overall resulted in decreased transport of flavonoids, though we were unable to detect metabolites in this model. Analysis of gene expression data of key transport and metabolizing systems revealed alterations with repeated blackberry exposure, indicating that the gut may potentially be a driver of differences observed in absorption and metabolism of flavonoids.

Though the mechanisms behind these adaptive phenomena are not well understood, it is known that the intestinal enterocytes have a high turnover rate and the nascent cells repopulating the epithelial lining can be influenced by the extracellular matrix (Reya and Clevers, 2005). Thus it is possible that dietary factors including flavonoids can influence the differentiating cell's function leading to altered bioavailability. Supporting this notion are *in vitro*, cell-based data demonstrating that Caco-2 cell monolayers undergoing repeated treatment with flavonoid-rich extracts of grape products during the differentiation process exhibit altered markers of differentiation (Laurent et al., 2004, 2005). Additionally, transport systems such as OAT (organic anion transporters) in liver tissue can be altered by long-term treatment of anthocyanins (Riha et al., 2015). Thus, there are likely alterations occurring at the molecular level over a 3-week period that may relate to the the observed differences in the absorption and metabolism of these flavonoids. Although the exact tissue-level

alterations are yet unknown, the gut and liver are potential candidates since they are major sites of metabolism and transport of these compounds.

In contrast to previous literature that observed adaptation to extracts rich in flavan-3-ols in rodent models (Ferruzzi et al., 2009; Wang et al., 2012), the present study did not observe significant differences in plasma AUC or urinary accumulation of epicatechin metabolites with repeated exposure to blackberry. Lack of an adaptive response may be due to differences food matrix, interactions between flavonoids, or phylogenetics. In addition, the differing doses need to be considered as the amount of epicatechin in blackberry (approximately 0.6 mg/g) was relatively low compared to that of grape seed extracts which can contain up to approximately 60 mg/g epicatechin (Villani et al., 2015). Still, because of the relatively small amount of participants in this study (n = 18) differences that are not significant should be taken with caution. The study appears to be statistical underpowered as a post-hoc power analysis reveals that at least 38 participants would be needed to detect significance differences in lean and obese cyanidin-3-*O*-glc appearance in urine.

Though anthocyanins are considered to have the lowest oral bioavailability of the flavonoids when solely considering the parent compound, in fact, they have a much higher systemic bioavailability (approximately 20%) when considering both Phase II and microbial metabolites of these compounds (Czank et al., 2013). In the present study, we focused on the parent compounds and their Phase II metabolites although some studies using animal models report that gut microbiota adapts with repeated exposure to flavonoids and other phenolics (Wang et al., 2015). These bacterial metabolites should

be considered in future studies as the production of these compounds likely changes in humans with repeated exposure to flavonoids.

Strengths of this study include its controlled, randomized, cross-over design. Also, possible variation in flavonoid absorption due to of differences in background diet were reduced through standardization of the diet in the three weeks leading up to the PK assessment. One of the study's limitations include its relatively short duration since broad dietary patterns are generally longer than this period of time. In addition, our small sample size may have decreased the ability to detect differences. Though we tracked parent flavonoids and their Phase II metabolites, shifts in gut microbial populations through diet modifications may result in increased production of low-molecular weight flavonoid catabolites that are not accounted for in the current analysis.

5.5 Conclusion

While pharmacokinetic assessments used in determining the bioavailability of flavonoids have typically been performed using a paradigm of acute oral doses, the current findings suggest that adaptation in absorption and metabolism of select flavonoids may occur over three weeks of repeated exposure to blackberry as part of a mixed diet. The present study showed that in lean but not obese individuals, plasma AUC of methylated derivatives (peonidin-glc) of cyanidin-3-*O*-glc increased after repeated exposure to blackberry. In addition, metabolism of flavonoids appeared to differ based on the BMI of the individual, as this study observed increased urinary accumulation of anthocyanin metabolites in lean but not obese individuals after

undergoing repeated exposure to blackberry. Further research should continue to characterize this adaptive effect to isolate specific molecular mechanisms and locations (gut, liver, etc) for these phenomena in order to better leverage these findings for the purposes of optimizing delivery and dietary recommendations consistent with prevention of chronic disease. These data also help inform recommendations for the increasingly common issue of obesity in both industrialized and even developing countries, since this population may have altered metabolism of flavonoids. Additionally, results suggest that further knowledge of adaptation to repeated exposure of flavonoid-rich foods may better inform our understanding of the true extent of the bioavailability of these compounds and dietary/physiological factors that impact these measures. Taken together, these data demonstrate that repeated exposure to flavonoid-rich foods more consistent with their inclusion in a longer term dietary patterns may lead to a different bioavailabilities for individual flavonoids compared to those observed from a single acute dose.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Overall Conclusion

Though consumption of foods rich in flavonoids including beverages and fruits such as tea, grape, and berries have been associated with decreased risk of various chronic diseases, application for chronic disease prevention has been argued to be limited since their acute oral bioavailability is low (less than 5%). Still, chronic dosing paradigms have been reported to increase systemic bioavailability of flavonoids in both animal (Ferruzzi et al., 2009; Wang et al., 2012) and human models (Chow et al., 2003). Characterization of the absorption and metabolism of flavonoid-rich foods and the factors that influence bioavailability are essential in order to correlate epidemiological observations to chronic disease preventative endpoints. This dissertation aimed to fill the knowledge gap with special focus on adaptive phenomena that influence bioavailability of flavonoids such that it can be leveraged for maximizing flavonoid bioavailability. More specifically, it aimed to model adaptive phenomena observed *in vivo* by utilizing an *in vitro*, cell-based model of the small intestinal epithelial cells in order to determine alterations occurring at the gut level from repeated exposure to flavonoids in addition to characterizing metabolites that result from xenobiotic metabolism of flavan-3-ols such as from tea and grape seed.

First, the circulating flavan-3-ol metabolite profile of rodents administered grape seed was elucidated through a combination of our collaborators supplying us with enzymatically-synthesized flavan-3-ols metabolites combined with our use of NMR and MS to characterize the structure of these compounds. To summarize, Chapter 2 concluded that the flavan-3-ols metabolites appearing in rodent plasma could be matched with enzymatically synthesized glucuronide and methyl glucuronide flavan-3-ol metabolites. By using 1D and 2D NMR spectroscopy, we showed that methylation occurs at the 3' position of both catechin and epicatechin and that major sites of glucuronides occur at the 5 and 7 positions. In addition, a two-site laboratory validation demonstrated that the analytical method we developed were robust across instruments and personnel. Interestingly, we also identified a large difference in the mass spectrometry analysis response factor between the parent compound and glucuronide metabolites of flavan-3-ols, which may lead to an overestimation of these metabolites in biological samples and is not regularly accounted for in these types of analyses.

Once the chemical structure of the circulating flavan-3-ol metabolites found in rodents administered grape seed extract was determined, we turned to the research performed in Chapter 3 that highlighted the effect of chronic exposure of the isolated flavan-3-ols EGCG and EC in addition to tea and grape seed extracts rich in flavan-3-ols on their intestinal transport using the Caco-2 intestinal cell culture model. We concluded that repeated low-dose (1-10 μM) exposure to these compounds and extracts during the cell differentiation period increased transport of these compounds and altered its metabolite profile. Interestingly, treatment with these extracts also

appeared to affect the barrier function of the cell model. Limitations of this model is that it considers only the gut as an agent of flavonoid bioavailability and metabolism, in addition to that metabolites were not detectable when using enriched extracts.

Given that chronic exposure to isolated flavan-3-ols and enriched extracts were able to affect their intestinal transport and metabolism, we turned to probing additional classes of flavonoids and phenolic acids by utilizing blackberry extract. We used a similar chronic dosing paradigm as described in Chapter 3 to test if low dose exposure resulted in differences in transport of the various phenolic compounds. We interestingly observed an overall decrease in the transport of the parent compounds with repeated exposure, though we were unable to detect the metabolites. Gene expression analysis of the xenobiotic metabolizing and transport systems indicated an overall decrease in a majority of these systems, which correlated to the decreases in transport of select flavonoids and phenolic acids. As in the limitations in the previous chapter, we were not able to detect metabolites using this model but the utility of this model is still apparent.

Taken together, Chapter 3 and 4 suggest that gut adaptation to flavonoids may be class specific and dependent on matrix and interactions between flavonoids. For instance, tea and grape contain largely monomeric and polymeric flavan-3-ols, while berries contain large amounts of anthocyanins, flavonols, and phenolic acids. This deserves further investigation as knowledge of differences in adaptation to specific classes of flavonoids and food helps determine factors that influence adaptive responses in humans.

Finally, we performed a clinical study to characterize *in vivo* adaptation by determining the effect of repeated 3-week exposure to blackberry fruit on the absorption and metabolism of flavonoids, in addition to determining any effect of BMI on these endpoints. Chapter 5 concluded that adaptation in bioavailability and metabolism in humans occurs after 3-week daily exposure to flavonoid-rich blackberry. The amount of blackberries administered in the study was 300 g or approximately 4 servings of fruit, which was divided between breakfast and dinner meals. The amount of berries given at the PK assessment (300 g) is approximately that recommended by the 2015 Dietary Guidelines for Americans. Anthocyanins, flavan-3-ol, and flavonol parent compounds and metabolites were the major flavonoids detected in circulation and found accumulated in urine. Plasma AUC of methylated cyanidin-3-*O*-glc derivatives increased after chronic exposure, possibly indicating induction of metabolizing enzyme systems. Interestingly, epicatechin-3'-*O*-glucr appearance in plasma or its accumulation in urine did not appear to be influenced by either blackberry exposure or BMI, which is in contrast to previous animal work. This may be due to several factors, including differences between humans and rodents, along with dosage and food matrix.

In conclusion, bioavailability of select flavonoids from blackberries appears to change based on previous exposure to these compounds, in addition, the adaptive response and absolute bioavailability of these compounds may be influenced by BMI. This is of interest because of the large increase in the incidence of obesity in both industrialized and developing countries and thus may affect recommendations for these compounds. However, for some specific classes of flavonoids, such as flavan-3-ols,

bioavailability and metabolism may not be as negatively affected compared to other classes. This may be a reflection of differences in specific transport and metabolizing systems these compounds undergo. When comparing results from the *in vitro* data from the Caco-2 model to the *in vivo* data, there are some correlation but also differences. Flavan-3-ols from the tea and grape chronic treatment displayed increases in transport, and pure compounds generally showed increases in metabolism. In contrast, results from blackberry treatment did diverge slightly from this, showing decreases in transport of parent compounds. However, this model was limited since we were not able to detect metabolites, so any shift in metabolism would not be captured.

6.2 Future Directions

Our data demonstrated that adaptation can occur in humans and that the gut likely plays a vital part in the alterations that occur with repeated exposure. While the data is promising, the speculation on the mechanism of whether adaptation in the absorption and metabolism of flavonoids in humans are entirely due to the gut needs further investigation. In addition, the effect of the obese condition on the mechanisms of flavonoid absorption and metabolism needs to be further characterized. It has been shown that impaired insulin signaling that can occur with obesity may alter expression of drug metabolizing enzymes responsible for glucuronidation and sulfation of flavonoid metabolites (Kim and Novak 2007). This change in activity of Phase II metabolizing enzymes is likely to alter the generation of conjugated flavonoid metabolites. It will be interesting to further explore whether the function of these xenobiotic systems are

compromised in obese populations. Future work can incorporate additional aspects of human physiology into our reductionist model of the small intestine epithelia to generate a more complex model since xenobiotic metabolism involves more than the gut, with the liver and kidney also playing integral roles. Finally, changes in the absorption and metabolism of these compounds is especially interesting in potential biomarkers of intake. If these markers produce robust responses and differences between individuals who are habitual versus sporadic consumers, there is more potential for candidate markers to be identified and used in large epidemiological studies.

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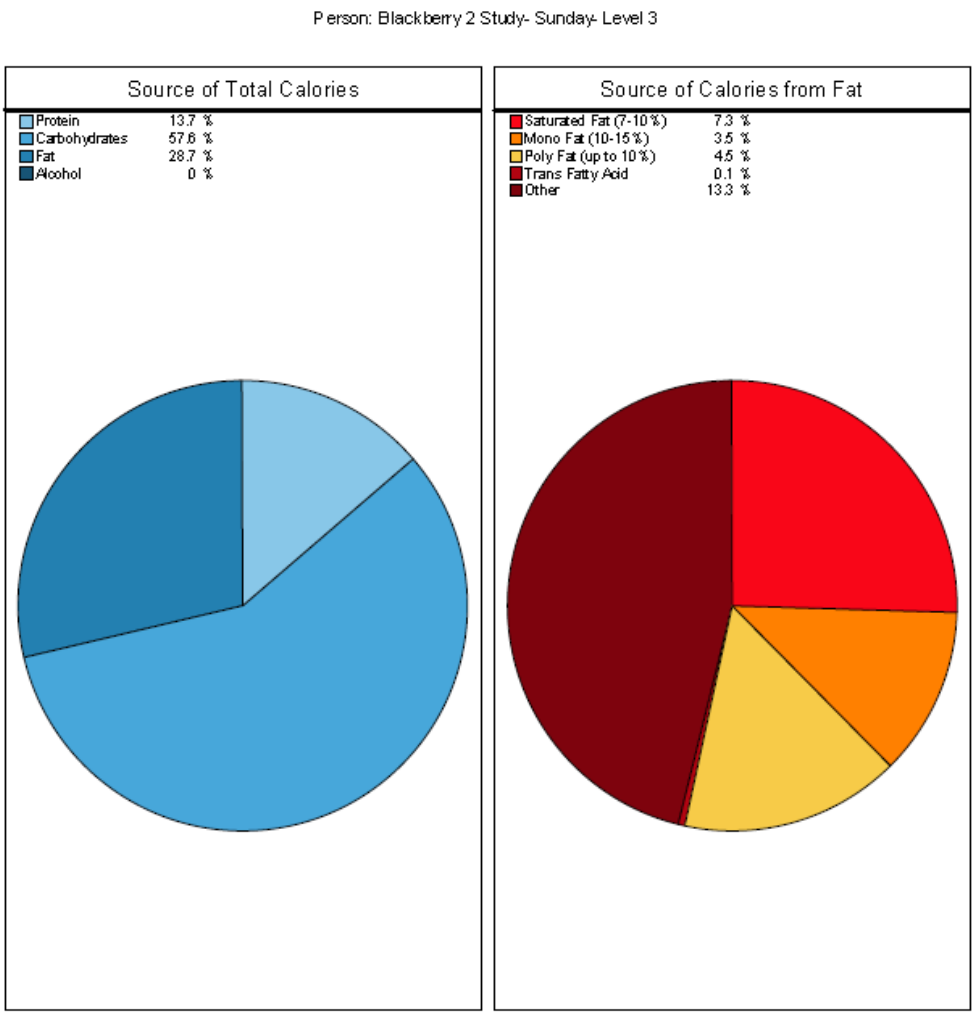
APPENDICES

Appendix A Weekly Meal Composition for Blackberry Study

Appendix Table A. 1. Example meal composition for study participants during controlled feeding periods.

Date:	Blackberry Microbiota Study- SUNDAY										
	CONTROL										
	TREATMENT										
INITIALS	Production Numbers (TOTAL)										
		L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
	Breakfast										
	Waffles- AUNT JEMIMA	60	67	73	80	86	94	100	107	113	120
	Promise Buttery Spread	6	7	7	8	9	9	10	11	11	12
	Syrup, pancake	18	20	22	24	26	28	30	32	34	36
	Sausage, turkey patty- Jimmy Dean	60	67	73	80	86	94	100	107	113	120
	Milk, 2%	128	142	156	170	184	200	214	228	242	256
	BLACKBERRY, FROZEN, UNSWEETENED	150	150	150	150	150	150	150	150	150	150
	STRAWBERRY JELLO	136	136	136	136	136	136	136	136	136	136
	Lunch										
	Kaiser Roll- Ottenbergs	56	62	68	74	81	87	94	100	106	112
	Beef BBQ- Brookwood Farms	80	89	98	106	115	125	134	142	151	160
	Avocado Study- Potato Salad	99	110	121	132	143	154	165	176	187	198
	Peaches, DOLE	80	89	98	106	115	125	134	142	151	160
	Dinner										
	Spaghetti- BARILLA	90	100	110	120	130	140	150	160	170	180
	Spaghetti Sauce w/meat- RAGU	70	78	85	93	101	109	117	125	132	140
	Mozzarella Cheese, Part-Skim, Shredded	18	20	22	24	26	28	30	32	34	36
	Vegetables, mixed, fzn	80	89	98	106	115	125	134	142	151	160
	Cool Whip, lite	10	11	12	13	14	16	17	18	19	20
	BLACKBERRY, FROZEN, UNSWEETENED	150	150	150	150	150	150	150	150	150	150
	STRAWBERRY JELLO	136	136	136	136	136	136	136	136	136	136
	Evening Snack				0						
	Celery, stalk	55	61	67	73	79	86	92	98	104	110
	Salad Dressing, ranch	32	36	39	43	46	50	53	57	60	64
	Cracker, cheese, gold fish	28	31	34	37	40	44	47	50	53	56

*Note: The “L” number is based upon the participant’s daily energy needs.



Appendix Figure A. 1. Macronutrient distribution of an example meal for study participants during controlled feeding periods.

Appendix B Flavonoid Stabilization Procedure for Urine and Plasma

Human Plasma acidification protocol for Stabilizing (non-anthocyanin) Flavonoids

- Freshly prepare 1% ascorbic acid/water (weight:volume) by dissolving 10 g L-ascorbic acid (catalogue# A92902, Sigma-Aldrich) into 1 L of nanopure water
- Aliquot 250 μL of ascorbic acid solution into each 2 mL EDTA-cyrovial, keeping vials cold on ice
- Aliquot 1 mL of plasma into vial with the ascorbic acid solution
- Transfer plasma to -80°C

Human Plasma acidification protocol for Stabilizing Anthocyanins

- Prepare a 12.5% (v/v) formic acid (catalogue# 695076 Sigma-Aldrich) solution by bringing 125 mL formic acid up to 1 L with nanopure water
- Aliquot 120 μL of 12.5% formic acid solution into each 2 mL EDTA-cyrovial, keeping vials cold on ice
- Aliquot 1 mL of plasma into vial with the formic solution
- Transfer plasma to -80°C

Human Urine acidification protocol for Stabilizing All Flavonoids

- Prepare a 1% (v/v) formic acid (catalogue# 695076 Sigma-Aldrich) solution by bringing 10 mL formic acid up to 1 L with nanopure water
- Dilute urine at a ratio of 1:5 1% formic acid:urine
- Transfer urine to -80°C

Appendix C IRB Consent Form for Blackberry Clinical Study

IRB number:	**Clinical Site IC Version:** Version 2
Project Title:	Blackberry Flavonoid Absorption and Effects on Intestinal Bacteria
Principal Investigator:	Janet A. Novotny **Institution:** USDA

MedStar Health Research Institute Informed Consent for Clinical Research

INTRODUCTION

We invite you to take part in a research study called "*Blackberry Flavonoid Absorption and Effects on Intestinal Bacteria.*" You were selected as a possible participant in this study because you applied for the study and you met the inclusion criteria. Please take your time to read this form, ask any questions you may have and make your decision. We encourage you to discuss your decision with your family, friends and your doctor(s).

WHAT IS THE PURPOSE OF THIS STUDY?

This study is being done to learn about the absorption of blackberry nutrients called flavonoids and their potential benefits in reducing risk of cancer.

WHAT ELSE SHOULD I KNOW ABOUT THIS RESEARCH STUDY?

It is important that you read and understand several points that apply to all who take part in our studies:

- Taking part in the study is entirely voluntary and refusal to participate will not affect any rights or benefits you normally have;
- You may or may not benefit from taking part in the study, but knowledge may be gained from your participation that may help others; and
- You may stop being in the study at any time without any penalty or losing any of the benefits you would have normally received.

The nature of the study, the benefits, risks, discomforts and other information about the study are discussed further below. If any new information is learned, at any time during the research, which might affect your participation in the study, we will tell you. We urge you to ask any questions you have about this study with the staff members who explain it to you and with your own advisors prior to agreeing to participate.

WHO IS IN CHARGE OF THIS STUDY?

The investigator is Janet Novotny, PhD. The research is being sponsored by the United States Department of Agriculture. MedStar Health Research Institute is being paid by the U.S. Department of Agriculture to conduct this study with Janet Novotny, PhD, as the primary investigator.

WHO CANNOT PARTICIPATE IN THIS STUDY?

You cannot be in this study if any of the following apply to you:

- Younger than 25 years old or older than 75 years old
- Use of blood-thinning medications such as Coumadin (warfarin), Dicumarol (dicumarol), or Miradon (anisinidione)
- Presence of any gastrointestinal disease, metabolic disease, or malabsorption syndromes that may interfere with the study goals

- Have been pregnant during the previous 12 months, are currently pregnant or lactating, or plan to become pregnant during the study
- Type 2 diabetes requiring the use of oral antidiabetic agents or insulin
- History of eating disorders or other dietary patterns which are not consistent with the dietary intervention (e.g., vegetarians, very low fat diets, high protein diets)
- Use of prescription or over-the-counter antiobesity medications or supplements (e.g., phenylpropanalamine, ephedrine, caffeine) during and for at least 6 months prior to the start of the study or a history of a surgical intervention for obesity
- Active cardiovascular disease (such as a heart attack or procedure within the past three months or participation in a cardiac rehabilitation program within the last three months, stroke, or history/treatment for transient ischemic attacks in the past three months, or documented history of pulmonary embolus in the past six months).
- Use of any tobacco products in past 3 months
- Unwillingness to abstain from herbal supplements for two weeks prior to the study and during the study
- Known (self-reported) allergy or adverse reaction to blackberries or other study foods
- Unable or unwilling to give informed consent or communicate with study staff
- Self-report of alcohol or substance abuse within the past twelve months and/or current acute treatment or rehabilitation program for these problems (long-term participation in Alcoholics Anonymous is not an exclusion)
- Other medical, psychiatric, or behavioral factors that in the judgment of the Principal Investigator may interfere with study participation or the ability to follow the intervention protocol

WHAT IF I AM PRESENTLY PARTICIPATING IN ANOTHER RESEARCH STUDY?

Are you presently participating in any other research studies? Yes ☐ No ☐

If yes, please state which study(ies)_____

While participating in this study, you should not take part in any other research project without approval from the people in charge of each study. This is to protect you from possible injury arising from such things as extra blood drawing, extra x-rays, interaction of research drugs, or similar hazards.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 48 people will take part in this study, worldwide. Forty-eight people will be recruited at this site.

WHAT HAPPENS IF I AGREE TO BE IN THE STUDY?

If you agree to take part in this study, you will be "randomized" into one of the study groups. Randomization means that you are put into a group by chance. It is like flipping a coin. Neither you nor the investigators will choose what group you will be in. You will have a one in two chance of being placed in any group. Your group assignment determines the order in which you will participate in the two parts of the study. Of the 48 participants, 28 will be selected to participate in 2 additional testing days (one at the end of each of the two study parts).

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Screening Procedures:

You will be scheduled for a brief screening at the Beltsville Human Nutrition Research Center (the Center). We will collect a blood sample of 25 ml (about 2 tablespoons) and a small urine sample. The blood sample will be analyzed for chemical compounds and for numbers of different types of blood cells. These analyses will include red and white blood cell counts, platelets, sugar, insulin, liver enzymes, bilirubin, cholesterol, and triglycerides. The urine sample will be used for routine laboratory analysis that includes testing for the presence of blood cells, sugar, or proteins. These are the same tests that a doctor uses to see if someone is healthy. You will also have your blood pressure tested. These tests and the physical exam will determine whether you are eligible for the study. You will be notified about any abnormal blood or urine test results and we advise that you follow up these results with your primary physician. These samples will be discarded after completion of the lab analyses. These results will be provided to you whether you are eligible for the study or not.

You will be selected to join based on health status, weight, body mass index, normal range of blood and urine analysis values, and availability to participate in the complete study. Your blood values for cholesterol, waist and hip size, blood triglycerides, blood sugar, and blood pressure will also be used for diet selection. If more people apply for the study than there are openings for participants, then participants will be selected to maximize our range of values for cholesterol, waist and hip size, blood triglycerides, blood sugar, and blood pressure. Of the 48 study participants, 28 will be selected to participate in 2 additional testing days. Individuals will be preferentially selected who fall into the upper and lower ends of BMI. Fourteen individuals will be selected for the additional testing days who have high BMI, and fourteen individuals will be selected for the additional testing days who have a low BMI.

Diet Procedures:

You will participate in two controlled feeding periods that will be 4 weeks each in length. During these periods, you will consume a typical American diet provided by the USDA Nutrition Center. During one of the diet periods you will consume blackberries as part of the controlled diet and during the other period you will consume jello (control) as part of the controlled diet.

For the controlled feeding, you will consume breakfast and dinner daily at the Center, and an early breakfast on the fourth and eighth day of the study. The dining facility is open from 6:30 to 8:30 AM for breakfast and from 4:30 to 6:30 PM for dinner. Lunch on all other days will be provided for carry-out. Meals will be prepared using traditional American foods. You will be expected to eat everything we provide for you, and not consume any other foods or beverages without approval from our staff. You will be expected to avoid food and beverages containing caffeine, except those provided by the Center. Alcohol consumption will not be allowed during the treatment period.

You are asked to limit intake of coffee to 2 cups per day or less during the treatment periods and you are asked to keep your coffee consumption constant. You also are asked to abstain from vitamin, mineral, and herbal supplements for the duration of the study. On the mornings of your blood draws, you are asked to refrain from vigorous exercise.

You will be fed enough food to keep your body weight the same during the study. Each day we will weigh you before breakfast. If your body weight begins to change, we will adjust the amount of food that you are eating.

If you agree to participate in this study you will be required to:

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1) Consume a controlled diet provided by the USDA Nutrition Center for 8 weeks. On weekdays, you will come to the Center for breakfast and dinner, and lunch will be given to you for carry-out. On Friday evening, you will take the food we provide home with you to consume on the weekend. You must eat all of the food that we give to you and you cannot eat any food that we do not give to you.

2) Come to the Center before the start and end of each treatment period to receive supplies to collect feces. You will collect one fecal sample in containers we provide at the beginning and end of the study.

3) Come to the Center at the beginning and end of each treatment period and provide a blood sample after a 12 hour fast.

4) You will provide twourine samples on two mornings of each feeding period (once during week 1 and once during week 2), at 0h and 3 hr after a test breakfast.

4) You may be selected to participate in 2 additional testing days. If selected, you will come in for an additional testing day at the end of each diet period. You will have your blood drawn, using a catheter, at the following times, before and after a test meal: -15, 0, 30, 60, 90, 120, 150, 180, 240, 300, 360, 420 minutes. You will also collect your urine on each of these 2 additional testing days, at the following times: 0, 90, 180, 270, 360, 420 minutes.

5) Be weighed daily on a bathroom type scale, prior to breakfast at the Center, during the controlled feeding diet period.

6) Fill out a daily questionnaire about your general health and exercise during the controlled feeding diet period. It will take about 5 minutes each.

Blood collection:
You will provide blood samples on at least four different study days. The amount of blood collected at the start and end of each treatment period will be approximately 4 tablespoons. If selected for the additional 2 testing days, an additional 6 tablespoons will be collected on each day. The total amount of blood collected over the entire study will be under 1 pint, less than what the Red Cross will allow for blood donation. The blood we collect will be analyzed for nutrients from blackberries, blood sugar, and markers of cancer disease risk and gut health. We will also test for genotype for body processes involved in absorption and metabolism of nutrients found in DNA, and we will investigate RNA (which is DNA's messenger) to learn what pathways your body turns up or down after consuming different dietary components. The genetic component of the study is not optional.

Other study procedures:

Please tell the investigator about all medications including over the counter drugs or herbal supplement you are taking, even if you don't think they are important.

You may be required to withdraw from the study for reasons that you cannot control, including illness.

We would like to keep your name and address on file to help recruit for future studies. Please sign below if you agree to allow us to keep this information on file for this purpose.

IRB Approval Stamp
(ORA USE ONLY - DO NOT CHANGE ANY INFORMATION IN THIS SECTION)
MedStar Health Research Institute
APPROVAL DATE JUL 2 1 2013
APPROVAL EXPIRES MAY 1 7 2014
IRB APPROVED Form Revised Date: 07/10/2012

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Participant's Signature _____ Date of Signature _____

The investigator may terminate your voluntary participation in this study if you do not follow the procedures listed above.

The treatments in this study that are considered experimental/investigational are the inclusion of blackberries in the diet to reduce risk of cancer.

HOW LONG WILL I BE IN THE STUDY?

You will be in the study for no longer than 14 weeks (including the break period).

The investigator may decide to take you off this study if it is believed to be in your best interest, you fail to follow instructions, new information becomes known about the safety of the study, or for other reasons the investigator or sponsor believes are important.

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the investigator and your regular doctor first so they can help you decide what other options may be best for your medical care once you are off study.

If you suddenly withdraw from the study, we may not be able to use any of the information gathered from your participation.

WHAT ARE THE RISKS AND SIDE EFFECTS OF THIS STUDY?

If you decide to participate in this study, you should know there may be risks. You should discuss these with the investigator and/or your regular doctor and you are encouraged to speak with your family and friends about any potential risks before making a decision. Potential risks and side effects related to this study include those listed below.

Risks and side effects ***that may occur*** include:

- Bruising from the blood draw

Risks and side effects ***that are less likely to occur*** include:

- Light-headedness during the blood draw
- Pain or discomfort during the blood draw

Risks and side effects ***that rarely occur*** include:

- Fainting during the blood draw
- Infection at the site of the blood draw

ARE THERE ANY BENEFITS TO TAKING PART IN THE STUDY?

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Principal Investigator:	Janet A. Novotny **Institution:** USDA

You may or may not get any direct benefit from being in this study. We cannot promise that you will experience any benefits from participating in this study. We hope the information learned from this study will benefit others in the future.

WHAT OTHER OPTIONS ARE THERE?

Instead of being in this study, you have these options:

- You always have the option to not be in this study or to refuse any medical treatment.

WHAT ABOUT CONFIDENTIALITY?

Your personal health information (PHI) will be kept private to the extent allowed by law. You will not be identified by name in any publications resulting from this study. Data and samples from this study will be disposed of after all publications concerning the study are completed. As required by the U. S. Department of Agriculture, consent forms and medical screening data will be kept for 25 years, and then destroyed. All other data, records, and samples will be kept until manuscripts have been published, and then they will be destroyed. Samples collected at screening will be destroyed immediately after analysis. If you do not wish to sign this permission form you will not be allowed to participate in this study.

Information, that does not include personally identifiable information, concerning this clinical trial has been or may be submitted, at the appropriate and required time, to the government-operated clinical trial registry data bank, which contains registration, results, and other information about registered clinical trials. This data bank can be accessed by you and the general public at www.ClinicalTrials.gov. Federal law requires clinical trial information for certain clinical trials to be submitted to the data bank.

WILL I BE PAID FOR PARTICIPATING IN THIS STUDY?

You will be paid for being in this study. You will receive $400 for completion of each treatment period, , including all meals and measurements, for a total of $800. If selected for the additional 2 testing days, participants will be paid an additional $200 for completion of each additional visit, for a total of $1200, for completion of all meals and measurements. Payment will be issued upon completion of the entire study. If there is evidence that you have not complied with the study protocol, it is possible that we will remove you from the study with no monetary compensation. Because this is a short study, there will be no partial compensation for completing only part of the study. Compensation for research participants is considered taxable income. Amounts of $600.00 or more will be reported to the Internal Revenue Service (IRS).

WHAT ARE THE COSTS?

You do not have to pay anything to be in this study. However, if taking part in this study leads to procedures or care not included in the study, it may lead to added costs for you or your insurance company. You will not be charged for any tests or procedures that are part of this research study.

WHAT IF I'M INJURED OR BECOME ILL DURING THE STUDY?

We will make every effort to prevent injuries and illness from being in the study. In the case of an injury, illnesses, or other harm occurring during, or resulting from, the study, emergency medical treatment is available but will be given at the usual

charge by an area hospital. You or your insurance company will be charged for any continuing medical care and/or hospitalization that are not a part of the study.

If you suffer an injury related to the study procedures, the reasonable costs of necessary medical treatment of the injury will not be reimbursed by the USDA to the extent these costs are not covered by your insurance or third party coverage. If you have an injury or illnesses occurring during, or resulting from the study, you, your medical insurance, a third-party payer, or a government program you've enrolled will be expected to provide coverage for your medical care. Federal government does not have any program to provide compensation to you if you experience injury or other bad effects that are not the fault of the investigators. If you are injured while participating in this research project as a result of the negligence of a United States Government employee who is involved in this research project, you may be able to be compensated for your injury in accordance with the requirements of the Federal Tort Claims Act. Compensation from individuals or organizations other than the United States might also be available to you. If you are a federal employee acting within the scope of your employment, you may be entitled to benefits in accordance with the Federal Employees Compensation Act.

No funds have been set aside, by the USDA, the MedStar Health Research Institute, MedStar Health, or its affiliated entities to repay you in case of injury, illness, or other harm occurring during, or resulting from the study and their current policies do not provide for payments for lost wages, cost of pain and suffering, or additional expenses. By agreeing to this you do not give up your rights to seek compensation in the courts.

WHAT ARE MY RIGHTS AS A PARTICIPANT?

- You have the right to be told about the nature and purpose of the study;
- You have the right to be given an explanation of the exactly what will be done in the study and given a description of potential risks, discomforts, or benefits that can reasonably be expected;
- You have the right to be informed of any appropriate alternatives to the study, including, if appropriate, any drugs or devices that might help you, along with their potential risks, discomforts and benefits;
- You have the right to ask any questions you may have about the study;
- You have the right to decide whether or not to be in the study without anyone misleading or deceiving you; and
- You have the right to receive a copy of this consent form.

By signing this form, you will not give up any legal rights you may have as a research participant. You may choose not to take part in or leave the study at any time. If you choose to not take part in or to leave the study, your regular care will not be affected and you will not lose any of the benefits you would have received normally. We will tell you about new information that may affect your health, welfare, or willingness to be in this study.

WHO DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, contact the investigator, Janet Novotny, PhD, at 301-504-8263. If you are having a medical emergency, you should call 911 or go directly to the nearest emergency room.

For questions about your rights as a research participant, contact the MedStar Health Research Institute. Direct your questions to the Office of Research Integrity at:

Address: MedStar Health Research Institute Telephone: (301) 560-2912

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6525 Belcrest Rd.
Suite 700
Hyattsville, MD 20782

Toll Free: (800) 793-7175
Fax (301) 560-7336

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IRB Approval Stamp
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MedStar Health Research Institute
APPROVAL DATE JUL 2 1 2013
APPROVAL EXPIRES MAY 1 7 2014
IRB APPROVED
Form Revision Date: 07/25/2010

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Principal Investigator:	Janet A. Novotny **Institution:** USDA

SIGNATURES

As a representative of this study, I have explained the purpose, the procedures, the possible benefits and risks that are involved in this research study. Any questions that have been raised have been answered to the individual's satisfaction.

Signature of Person Obtaining Consent _____ Date of Signature _____

Printed Name of Individual Obtaining Consent _____

I, the undersigned have been informed about this study's purpose, procedures, possible benefits and risks, and I have received a copy of this consent. I have been given the opportunity to ask questions before I sign, and I have been told that I can ask other questions at any time. I voluntarily agree to be in this study. I am free to stop being in the study at any time without need to justify my decision and if I stop being in the study I understand it will not in any way affect my future treatment or medical management. I agree to cooperate with Janet Novotny, PhD, and the research staff and to tell them immediately if I experience any unexpected or unusual symptoms.

Participant's Signature _____ Date of Signature _____

Printed Name of Participant: _____

As the Principal Investigator (or his designee) for this research study, I have reviewed this individual's eligibility for enrollment in the study and agree that the individual is eligible to be enrolled subject to results of screening.

Principal Investigator's Signature _____ DATE _____

VITA

VITA

Benjamin W. Redan

EDUCATION

Ph.D., Nutritional Biochemistry 2012–2016
Interdepartmental Nutrition Program, Advisor: Mario Ferruzzi
Purdue University, West Lafayette, IN

B.S., Biochemistry, *magna cum laude* 2007–2011
The University of Scranton, PA

POSITIONS HELD

Graduate Student Research Fellow 2012–2016
Phytochemistry & Bioavailability Laboratory, Purdue University,
Departments of Nutrition Science and Food Science, West Lafayette, IN

Post-Baccalaureate Fellow 2011–2012
National Institutes of Health (NIH), National Center for Advancing Translational Sciences, NIH Chemical Genomics Center, Rockville, MD

Laboratory Technician 2010–2011
Sanofi Pasteur, Co-operative in the Analytical Biochemistry
Platform of Research & Development, Swiftwater, PA

Undergraduate Student Research Fellow Summer 2009
University of Scranton, President's Fellowship for Summer Research

HONORS & AWARDS

National Science Foundation (NSF) Graduate Research 2013–2016
Fellowship, Life Sciences field of study

Outstanding Poster, National Confectioners Association's Chocolate Council 2014

Graduate Student Travel Award, American Society for Biochemistry and Molecular Biology, Experimental Biology National Meeting 2013

Travel Award, Purdue University Graduate Student Government 2013

Lynn Fellowship & Mary E. Fuqua Scholarship, Purdue University 2012–2013

Undergraduate Research Symposium Finalist, Agricultural and Food Chemistry Division of the ACS. Included travel award to present research in Boston, MA 2010

PRESENTATIONS

Redan B and Ferruzzi M. Differentiated Caco-2 Cell Monolayers Exhibit Differential Adaptation to Chronic Exposure of Green Tea and Grape Seed Extracts Rich in Flavan-3-ols (Oral Presentation) Experimental Biology, Boston, MA 04/2015

Redan B and Ferruzzi M. Chronic Flavan-3-ol Exposure Induces Changes in Metabolism and Transport Kinetics in Differentiated Caco-2 Cell Monolayers (Oral Presentation) Experimental Biology, San Diego, CA 05/2014

PROFESSIONAL DEVELOPMENT

Student Rep., Dietary Bioactives RIS, American Society for Nutrition 2014-2015

Attendee, Mary Frances Picciano Office of Dietary Supplements Practicum 06/2014

PUBLICATIONS

Redan B, Buhman K, Novotny J, Ferruzzi M. Altered Transport and Metabolism of Phenolic Compounds in Obesity and Diabetes: Implications for Functional Food Development and Assessment, (In review in *Advances in Nutrition*)

Carreiro A, Dhillon J, Gordon S, Jacobs A, Higgins K, McArthur B, **Redan B**, Rivera R, Schmidt L, Mattes R. The Macronutrients, Appetite, and Energy Intake, *Annu. Rev. Nutr.*, *36*, 73-103: 2016.

Blount J, **Redan B**, Ferruzzi M, Reuhs B, Cooper B, Harwood J, Shulaev V, Pasinetti, Dixon R. Synthesis and Quantitative Analysis of Plasma-targeted Metabolites of Catechin and Epicatechin, *J. Agric. Food Chem.*, *63*, 2233-2240: (2015).

D'Imperio M, Cardinali A, D'Antuono I, Linsalata V, Minervini F, **Redan B**, Ferruzzi M. Stability-activity of Verbascoside, a Known Antioxidant Compound, at Different pH Conditions, *Food Res. Int.*, *66*, 373-378: (2014).

Redan B, Vinson J, Coco Jr M. Effect of Thermal Processing on Free and Total Phenolics in Nine Varieties of Common Beans, *Int. J. Food Sci. Nutr.*, *64*, 243-247: (2013).